In Vitro and In Vivo Evidence for Anti-Inflammatory Properties of 2-Methoxyestradiol


Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia

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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 antiproliferative or antiangiogenic actions. Because 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 tumor necrosis factor-α production (by 60% at 3 μM). Estradiol had no effect on J774 macrophage activation, nor did the estrogen receptor antagonist 7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl][nonyl]estra-1,3,5(10)-triene-3,17β-diol (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). In addition, 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 the 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 neovascularization and growth of solid tumors (Fotsis et al., 1994). The antiproliferative and antiangiogenic actions of 2MEO have since been characterized in a variety of cell types and in vivo tumor 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 because of 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 antiproliferative and antiangiogenic actions of 2MEO on tumor and endothelial cells have been investigated extensively (Mooberry, 2003). 2MEO disrupts microtubule function, inhibits hypoxia-inducible factor 1α activity and cell cycle progression into S phase, and activates a number of proapoptotic signaling pathways (Hughes et al., 2002; Sutherland et al., 2007). 2MEO is also a low-affinity agonist for the estrogen receptor (Kd ~100 nM; compare 17β-estradiol Kd ~0.2 nM). However, the plasma levels of 2MEO required for anti-tumor 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 has yet to be determined (Ho et al., 2006).

In addition to anti-tumor 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 antiangiogenic 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) have reported 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 tumor 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 defense and play a central role in the inflammatory response. Activation by stimuli such as lipopolysaccharide (LPS) and interferon-γ results in the production of inflammatory signaling mediators that include nitric oxide, a free radical with antimicrobial activity, generated by inducible nitric-oxide synthase-2 (NOS-2); prostaglandin E₂ (PGE₂), a locally acting vasodilator generated by cyclooxygenase (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 characterize the effects of 2MEO on macrophage function in vitro and 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/IFN-γ-induced activation of the J774 murine macrophage cell line and human monocytes and suppresses acute LPS-induced airway inflammation in mice.

Materials and Methods

Mouse Macrophage Cell Line Culture and Treatments. The J774 A.1 murine macrophage cell line (American Type Culture Collection, Manassas, VA) was cultured in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% (v/v) heat-inactivated fetal 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 six-well plastic culture plates at a density of 4.0 × 10⁶ 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 six-well plates was considerably less than in 24- or 96-well plates, it was found that cells became overconfluent if plated at higher densities. Cells were grown to 90 to 95% confluence over 48 h before the culture medium was replaced with fresh medium (2 ml/well on six-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 an additional 2 h before treatment. Before stimulation with LPS (1 μg · ml⁻¹) and IFNγ (0.1 IU · ml⁻¹), cells were pretreated with 2MEO, 17β-estradiol, dexamethasone (DEX), colchicine, paclitaxel, and/or 7-[9-(4,4,5,5,5-pentafluoropropyl)sulfinyl]nonyl]estr-1,3,5(10)-triene-3,17β-diol (ICI 182,780). Unstimulated control groups were included in all experiments. The final concentration of dimethyl sulfoxide vehicle in the culture medium was controlled across all treatment groups ≤1.0% (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. After dextran sedimentation to minimize erythrocyte contamination (0.6% dextran T500 in saline for 1 h), buffy coat cell suspensions were washed twice in saline at 210g before layering over a Lymphoprep gradient and centrifugation for 20 min at 470g at room temperature without braking. The peripheral blood mononuclear cell layer was removed, resuspended to 50 ml in saline, washed three times at 210g, then resuspended in RPMI medium 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 15 mM HEPES, 2 mM L-glutamine, nonessential 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 × 10⁶ cells · cm⁻² and allowed to adhere for 2 to 3 h at 37°C in a humidified atmosphere containing 5% CO₂, after which nonadherent cells were removed by four vigorous washings in prewarmed saline. The remaining monocytes (average 0.5 × 10⁶ cells · cm⁻²) were incubated for 22 h before 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 11β-(p-dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)estradi-4,9-dien-3-one (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; Purina, St. Louis, MO) 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 (Szaroka et al., 1997). In brief, female BALB/c mice (9–11 weeks old) were anesthetized with sodium pentobarbital and tracheostomized. After tracheal intubation, the animals were ventilated using a Harvard 650 rodent respirator at a frequency of 120 breaths · min⁻¹ and tidal volume of 1 ml · kg⁻¹ body mass, with the exception of time course experiments, in which the frequency of ventilation was reduced to 70–80 breaths · min⁻¹. The end tidal CO₂ was maintained at 5–6% (v/v) and the inspired oxygen fraction was held constant at 0.14 (v/v). LPS (0.025 mg · kg⁻¹ body mass) was administered intratracheally in sterile saline (1 ml · kg⁻¹ body mass). Every 24 h, the mice were killed by overdose of pentobarbital. The lungs were weighed and fixed in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 24 h. After fixation, the lung tissues were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin for morphometric analysis of lung injury. The inflammation score was calculated as described previously (Szaroka et al., 1997).
weeks old; 20–25 g) in weight-matched treatment groups were lightly anesthetized with methoxyflurane by inhalation, before transnasal instillation of 1 μg of LPS in 35 μl of 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% dimethyl sulfoxide). 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. After 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 hemocytometer (Hawkesley, Sussex, UK), 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 was collected and stored at −20°C until protein concentrations were measured using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA), 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 OptEIA ELISA sets (BD-Australia/New Zealand, North Ryde, NSW, Australia) or matched antibody pairs according to the manufacturer’s protocols. The limits of detection for mouse IL-6 and TNF-α were 32 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 50 mM Tris-HCl) were incubated overnight at 4°C with [³H]PGE₂ (37 Bq/µl) and anti-PGE₂ (sufficient to give 30% maximal binding). The assay volume was 400 μl. Unbound [³H]PGE₂ was removed with 500 μl of ice-cold dextran-coated charcoal (20 μg · ml⁻¹ charcoal in assay buffer containing 4 mg · ml⁻¹ heat-dissolved dextran) and subsequent centrifugation (10 min, 1800 × g). The supernatants, containing the bound [³H]PGE₂, were mixed with 4 ml of 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 dpm) 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% naphthylethenediamine in dH₂O and 1% sulfanilamide in 5% (v/v) phosphoric acid, mixed 1:1 immediately before use) to 100 μl of undiluted cell supernatant samples. The absorbance at 550 nm was measured using a microplate reader (Multiskan Ascent, Thermo Fisher Scientific). Total RNA was isolated from a portion of the homogenized tissue by passing the tissue through a 21-gauge needle 5 to 10 times. Total RNA was isolated from a portion of the homogenized tissue by passing the tissue through a 21-gauge needle 5 to 10 times. The extracted RNA was eluted into 50 μl of water, and plates were air-dried for 1 h. The bound dye was solubilized in 10% acetic acid (100 μl/well) by gentle shaking for 1 min. The absorbance at 595 nm was measured using a microplate reader (Multiskan Ascent, Thermo Fisher Scientific). Cell number was calculated by comparison of sample absorbances with those of cell number standards (0–400,000 cells/well, seeded 4–6 h before fixing). A linear relationship between cell number and absorbance was observed over the range from 80,000 to 400,000 cells/well.

**Tissue RNA Extraction and Real-Time Polymerase Chain Reaction.** Real-time PCR was used to measure IL-6, TNF-α, COX-2, and NOS-2 mRNA expression. Mice lungs were pulverized in an RNase-free mortar and pestle under liquid nitrogen and homogenized by passing the tissue through a 21-gauge needle 5 to 10 times. Total RNA was isolated from a portion of the homogenized tissue using QIAGEN (Valencia, CA) RNeasy mini kits, according to the manufacturer’s instructions. The extracted RNA was eluted into 50 μl of 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 of 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 of diluted cDNA, 2.5 μl of Platinum SYBR Green qPCR Supermix-UDG, and 0.05 μl 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). The threshold cycle value determined for each gene was normalized against that obtained for 18S ribosomal RNA, which was included as an internal control.

**Data Analyses.** Results are expressed as mean ± S.E.M. 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 analyzed using Prism version 5.0b (GraphPad Software Inc., San Diego, CA). In most cases, a one-way analysis of variance (ANOVA) was conducted and treatment groups were compared with 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.** 2-MEO, 17β-estradiol, dexamethasone, paclitaxel, and ICI 182,780 were dissolved in 100% dimethyl sulfoxide to a stock concentration of 10 mM and stored at −20°C until required. Lyophilized 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. Lyophilized 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); Lynphoprep was from Axis-Shield (Oslo, Norway); mouse IL-6 and TNF-α OptEIA ELISA sets and human IL-6 and TNF-α-matched antibody pairs were from Becton Dickinson (North Ryde, NSW, Australia); protein assay kit and semi-dry transfer apparatus were from Bio-Rad Laboratories (Gladsvale, NSW, Australia); sheep anti-mouse IgG antibody and sheep anti-rabbit IgG antibody were from Abcam (Cambridge, UK); H[3]PGE₂, dextran T500, ECL reagent, and nitrocellulose membranes were from GE Healthcare (Rydalmere, NSW, Australia); Dulbecco’s Modified Eagle’s medium and RPMI 1640 medium, Superscript VILO cDNA synthesis kits, and all RT-PCR reagents were from Invitrogen (Mulgrave, VIC, Australia); fetal calf serum was from JRH Biosciences (Brooklyn, VIC, Australia); methoxyflurane was from Medical Developments International (Springvale, VIC, Australia); sodium pentobarbital was from Merial (Parramatta, NSW, Australia); ethidium bromide and acridine orange were from Invitrogen; scintillation fluid was from PerkinElmer (Glen Waverley, VIC, Australia); RNases Mini kits were from QIAGEN (Doncaster, VIC, Australia); recombinant mouse interferon-γ was from R&D Systems (Minneapolis, MN); L-glutamine was from SAFC Biosciences (Castle Hill, NSW, Australia); anti-PGE₂, bovine serum albumin, colchicine, crystal violet, dexamethasone, dextran, 17β-estradiol, HEPES, PLS (Escherichia coli serotype 0111:B4), nonessential amino acid solution, paclitaxel, penicillin/streptomycin solution, PGE₂, protease and phosphatase inhibitor cocktails, sodium bicarbonate, and sodium pyruvate were from Sigma-Aldrich (Castle Hill, NSW, Australia); 2-MEO was from Steraloids (Newport, RI); plastic culture plates were from NUNC (Roskilde, Denmark); ICI 182,780 was from Tocris Biosciences (Ellisville, MO); and the NOS-2 rabbit polyclonal antibody was from Transduction Laboratories (Lexington, KY).

**Results**

Lipopolysaccharide and Interferon-γ Induce J774 Macrophage Activation. 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-h 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 (Fig. 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 more than 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.** Because changes in cell number might confound the interpretation of measures of macrophage function, we examined the impact of LPS/IFNγ stimulation and 2-MEO treatment on J774 cell number at the 24-h time point. 2-MEO (10 µM) had no detectable effect on unstimulated cells; however, stimulation with LPS/IFNγ significantly lowered cell number (Table 2). Treating LPS/IFNγ-stimulated cells with 2-MEO (1–10 µM) caused no further reduction in cell number.

**2-Methoxyestradiol Suppresses J774 Macrophage Activation to an Extent Comparable with That of Dexamethasone.** When added 30 min before stimulation, 2-MEO (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 0.1 mM (Fig. 2). 2-MEO had no effect on LPS/IFNγ-induced TNF-α or nitrite production. These effects were observed with those of an established anti-inflammatory agent, the glucocorticoid dexamethasone, at 0.1 µM. This concentration of dexamethasone was more 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 (by 60%) production, but did not affect nitrite production. Neither 2-MEO (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, 2-MEO inhibited COX-2 protein expression (by 32% at 10 µM 2-MEO) but had no effect on NOS-2 protein expression (Fig. 2).

**17β-Estradiol Has No Effect on J774 Macrophage Activation.** Because 2-MEO 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 tumor epithelial cells (Sutherland et al., 2005), we examined the role of estrogen receptors in the action of 2-MEO on macrophage activation. 17β-estradiol has more than 100-fold greater affinity for estrogen receptors than 2-MEO and would be expected to saturate these receptors at
The Estrogen Receptor Antagonist ICI 182,780 Does Not Prevent the Effect of 2-Methoxyestradiol. The estrogen receptor antagonist ICI 182,780 \((K_i \sim 0.4 \text{ nM for both } \alpha \text{ and } \beta \text{ receptor subtypes})\) 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 \(\mu\)M, a concentration sufficient to saturate estrogen receptors and outcompete binding of the lower-affinity ligand 2MEO) did not affect LPS/IFN-\(\gamma\)-induced inflammatory mediator production in the absence of other treatments, nor did it prevent the suppression of LPS/IFN-\(\gamma\)-induced IL-6 and PGE\(_2\) production by 2MEO (Table 4). It is noteworthy that in the presence of ICI 182,780 the suppression of IL-6 production by 2MEO seemed to be modestly enhanced, and 2MEO inhibited LPS/IFN-\(\gamma\)-induced TNF-\(\alpha\) 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 polymerization by binding to free tubulin, whereas paclitaxel stabilizes microtubules by binding to the \(\beta\)-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 \(\mu\)M) inhibited LPS/IFN-\(\gamma\)-induced nitrite production (by up to 15 and 19\%, respectively) while enhancing LPS/IFN-\(\gamma\)-induced PGE\(_2\) production (by up to 159 and 60\%, respectively) (Fig. 3). Colchicine (but not paclitaxel) inhibited LPS/IFN-\(\gamma\)-induced IL-6 production by up to 34 and 40\%, respectively. In addition, colchicine (10 \(\mu\)M) increased PGE\(_2\) and nitrite production (by 78 and 14\%, respectively) in cells not stimulated with LPS/IFN\(\gamma\) (Table 3).

2-Methoxyestradiol Suppresses Activated Human Monocyte TNF-\(\alpha\) Production to an Extent Comparable to That of Dexamethasone. 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 \(\mu\)M) inhibited LPS/IFN-\(\gamma\)-induced TNF-\(\alpha\) production by up to 60\% at a concentration of 3 \(\mu\)M (Fig. 4). In cells from the same donors, dexamethasone (0.1 \(\mu\)M) inhibited TNF-\(\alpha\) production.
Effect of 2MEO, 17β-estradiol, dexamethasone, ICI 182,780, colchicine, and paclitaxel on basal inflammatory mediator production by unstimulated J774 macrophages

Table 3

<table>
<thead>
<tr>
<th>Treatment (Unstimulated Cells)</th>
<th>IL-6 (% LPS + IFNγ Response)</th>
<th>TNF-α (% LPS + IFNγ Response)</th>
<th>PGE2 (% LPS + IFNγ Response)</th>
<th>Nitrite (% LPS + IFNγ 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>

* P < 0.05, ** P < 0.01 compared with vehicle response (paired t tests).

Table 4

Effect of 2MEO on inflammatory mediator production by LPS/IFNγ-stimulated J774 macrophages in the presence and absence of ICI 182,780

<table>
<thead>
<tr>
<th>Treatment (LPS/IFNγ-Stimulated Cells)</th>
<th>IL-6 (% LPS + IFNγ Response)</th>
<th>TNF-α (% LPS + IFNγ Response)</th>
<th>PGE2 (% LPS + IFNγ Response)</th>
<th>Nitrite (% LPS + IFNγ 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 μM</td>
<td>88 ± 5</td>
<td>89 ± 8</td>
<td>73 ± 6</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>2MEO, 10 μM</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>

* P < 0.01 compared with LPS + IFNγ response.

† P < 0.05, ‡ P < 0.01, compared with LPS + IFNγ response in the presence of ICI 182,780 (one-way ANOVA with repeated measures, Dunnett’s post-test).
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. To investigate whether macrophage inhibition by 2MEO could be mediated via glucocorticoid receptors, we pretreated 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 macrophage 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 h (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 (Fig. 5). The effect of 150 mg · kg⁻¹ 2MEO was comparable with 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). Likewise, 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% (Fig. 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 after 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 antiangiogenic or antiproliferative 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) with 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 through those 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 antiproliferative or antiangiogenic actions, at doses similar to those having anti-tumor 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 macro-
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, 2 h), and/or 2MEO (3 μM, 30 min). Cell supernatants were collected 24 h after stimulation for measurement of TNF-α concentrations. Data, expressed as a percentage of the response in vehicle-treated cells, are presented as mean ± S.E.M. (n = 4). The concentration of TNF-α for the LPS + IFNγ response was 2.1 ± 0.9 pg · ml⁻¹.

<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, 3 μM</td>
<td>76 ± 11*</td>
</tr>
<tr>
<td>2MEO + RU486</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>
</tbody>
</table>

* P < 0.05 compared with LPS + IFNγ response.
† P < 0.05 compared with LPS + IFNγ response in the presence of dexamethasone (paired t test).

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 more than 95% (Maran et al., 2006). However, we did not detect significant changes in cell number after 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 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).
Effect of 2MEO and dexamethasone on LPS-induced increases in BAL cell number in mice and on TNF-

TABLE 6

<table>
<thead>
<tr>
<th>Treatment (Administered 2 h After LPS Instillation)</th>
<th>BAL Cell Number ($\times 10^6$)</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 ± 0.15</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>

Fig. 5. Effect of 2MEO and DEX on LPS-induced increases in BAL protein in mice and on IL-6 gene expression in mice 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$^{-1}$). A, 22 h later, mice were killed, and after BAL fluid collection for protein measurement, their lungs were removed. B, lung RNA was extracted and mRNA expression for IL-6 was determined by RT-PCR, relative to expression of the internal control 18S rRNA. Data are expressed as mean ± S.E.M. (n = 9–14). *, P < 0.05. **, P < 0.01 compared with LPS/vehicle response (one-way ANOVA, Dunnett’s post-test).

required for biological activity as a glucocorticoid (Axelrod, 1976).

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 destabilize microtubules by binding to the colchicine binding site of tubulin ($K_d$ ~20 μM) (D’Amato et al., 1994). Microtubule-interfering agents such as colchicine and paclitaxel are cytotoxic to proliferating cells (Molloinedo and Gajate, 2003) and modulate macrophage function (Mantovani, 1982). Although 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 PGE$_2$ 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.

Although 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 the 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 macrophages, they were not readily explained by a microtubule-related mechanism.
rophages 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 down-regulation of the hypoxia-inducible factor 1α, resulting in reduced expression of vascular endothelial growth factor, a proangiogenic 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 (Bozino

cell proliferation and blood vessel development. The molecu-
lates monocyte and macrophage activation in vitro and has

small half-life. We have shown that in BALB/c mice receiving

References


Inflammation showing less sensitivity to regulation by glucocor-

Acute anti-inflammatory activity in vivo. These findings pro-

generation a novel anti-inflammatory drug class (Hughes et al.,

such as LPS-induced neutrophilia or infiltrating monocytes) in addition to changed gene expression levels in resident cells such as alveolar macrophages.

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 contribut-

In the LPS-induced lung injury model, drugs were admin-

the lungs (such as LPS-induced neutrophilia or infiltrating monocytes) 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 · day⁻¹ 2MEO daily for 16 days serum levels peak at 100 to 400 nM (Sutherland et al., 2005). However, doses as low as 15 mg · kg⁻¹ · day⁻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 estrogene-

2002). Such optimization needs to be guided, among other things, by further elucidation of the mechanisms by which 2MEO modulates macrophage function (Sutherland et al., 2007).

In conclusion, our results demonstrate that 2MEO modu-

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Authorship Contributions

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

Contributed experiments: Shand, Langenbach, Kenan, Ma, Wheaton, Schuliga, and Stewart.

Performed data analysis: Shand, Kenan, and Schuliga.

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

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Address correspondence to: Alastair Stewart, Department of Pharmacology, University of Melbourne, Parkville, Victoria 3010 Australia. E-mail: astew@unimelb.edu.au