Prostaglandin E₂ Is a Novel Inducer of Oncostatin-M Expression in Macrophages and Microglia

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Oncostatin-M (OSM), a pluripotent cytokine of the interleukin-6 (IL-6) family, is produced in a number of inflammatory conditions. Known sources of OSM include monocytes–macrophages and T-cells. Here we present microglia, the resident macrophages of the brain, as a source of OSM in the CNS. In this context, we describe a novel inducer of OSM, prostaglandin E₂ (PGE₂). PGE₂ induces OSM expression in microglia, monocytes, and macrophages of human and murine origin. PGE₂ induction of OSM is mimicked by cholera toxin, an activator of stimulatory G (G_s)-proteins; by forskolin, an activator of adenylate cyclase; and by the cAMP analog, dibutyryl-cAMP. PGE₂ induction of OSM gene expression is inhibited by the adenylate cyclase inhibitor 2',5'-dideoxyadenosine, by the protein kinase A (PKA) inhibitor H-89, and by a dominant-negative PKA construct. These data indicate that PGE₂ signals via G_s-protein-

coupled receptor(s), adenylate cyclase, and PKA to induce OSM expression. Accordingly, other activators of cAMP signaling such as norepinephrine and PGE₁ induce OSM. The ability of PGE₂ to induce OSM expression was tested under more physiological conditions, using cocultures of astrocytes and monocytes. Treatment of the cocultures with IL-1 β or tumor necrosis factor- α (TNF- α) results in production of PGE₂ and OSM. PGE₂ produced in the cocultures is responsible for OSM induction, because pretreatment with indomethacin, an inhibitor of prostaglandin synthesis, as well as depletion of PGE₂, abrogate OSM expression induced by IL-1 β or TNF- α . These data suggest that in the CNS, OSM may be produced through collaboration of astrocytes and macrophages–microglia.

Key words: oncostatin-M; prostaglandin E₂; microglia; macrophage; astrocytes; cAMP

Oncostatin-M (OSM) is a cytokine of the interleukin-6 (IL-6) family, discovered in 1986 in the supernatants of the human monocytic cell line U937 treated with phorbol myristate acetate (PMA) (Zarling et al., 1986). OSM production has also been reported in T-cells (Brown et al., 1987; Radka et al., 1993), neutrophils (Grenier et al., 1999), and other cell types, but monocytes and macrophages remain the best source of this cytokine. Known inducers of OSM expression include physiological stimuli such as granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-3 (Ma et al., 1999), and human chorionic gonadotropin (hCG) (Komorowski et al., 1997), as well as pharmacological (PMA and cisplatin) (Sodhi et al., 1997) and viral (HIV-1) agents (Ensoli et al., 1999).

OSM exerts a number of biological effects in the CNS. OSM has been implicated as being responsible for neuronal apoptosis in HIV-1-associated dementia (Ensoli et al., 1999). OSM powerfully stimulates astrocytic production of IL-6 (Van Wagoner et al., 2000) and α_1 -antichymotrypsin (Kordula et al., 1998) and also regulates differentiation of astrocytes (Yanagisawa et al., 1999) and oligodendrocytes (Vos et al., 1996). In human cerebral endothelial cells, OSM upregulates expression of intercellular ad-

hesion molecule-1, IL-6, and the chemokine monocyte chemoattractant protein (MCP-1) (Ruprecht et al., 2001). In the periphery, OSM modulates a number of genes involved in inflammation, which suggests that it may play a modulatory role in the CNS inflammatory setting as well. We hypothesized that OSM is produced in the CNS by microglia, the resident macrophages of the brain. This is based on the common origin of microglia and macrophages during development (Ling, 1981) and the fact that macrophages are the best known source of OSM thus far.

Prostaglandin E₂ (PGE₂) is the most abundant prostaglandin in the brain, produced by almost all cell types present in the CNS (Katsuura et al., 1989; Rettori et al., 1992; Nogawa et al., 1997; Vegeto et al., 2001; Yamagata et al., 2001) after stimulation with inflammatory cytokines such as IL-1 β and tumor necrosis factor- α (TNF- α), as well as the bacterial lipopolysaccharide (Levi et al., 1998). Because it is rapidly metabolized, PGE₂ is thought to act locally by signaling through G-protein-coupled receptors located on the cell surface. To date, four types of PGE₂ receptors have been identified (EP1–EP4) (Narumiya and FitzGerald, 2001). Of these, EP2 and EP4 are coupled to stimulatory G (G_s)-proteins, which after PGE₂ binding lead to the activation of adenylate cyclase (Narumiya et al., 1999). This, in turn, increases intracellular cAMP levels and activates cAMP-dependent protein kinase (PKA), ultimately resulting in the transcription of cAMP-responsive genes.

Here we report for the first time that OSM is a cAMP-responsive gene and present PGE₂ as a novel inducer of OSM expression in cells of monocytic lineage. Furthermore, we establish microglia as a source of OSM and propose a collaborative model consisting of astrocytes and microglia–macrophages that may operate *in vivo* to produce OSM in the CNS.

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MATERIALS AND METHODS

Reagents. Recombinant human IL-1 β and TNF- α , as well as ELISA kits for OSM, cAMP, and PGE₂, were purchased from R & D Systems (Minneapolis, MN). Prostaglandin E₂, prostaglandin E₁, norepinephrine, cholera toxin (CTX), forskolin (FSK), 2',5'-dideoxyadenosine (DDA), dibutyl-cAMP (dbcAMP), and H-89 were purchased from Calbiochem (La Jolla, CA). Dimethylsulfoxide (DMSO) was purchased from Sigma (St. Louis, MO). Anti-PGE₂-coated Sepharose beads were purchased from Cayman Chemical (Ann Arbor, MI). Indomethacin was obtained from Biomol (Plymouth Meeting, PA).

Cell lines and primary human monocyte-derived macrophages. The human monocytic cell line THP-1 and the human astrogloma cell line CRT-MG were grown in complete RPMI 1640 medium [RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated fetal bovine serum (HI-FBS)], as described previously (Lee et al., 1997). In addition, the THP-1 medium was supplemented with 2 μ M 2-mercaptoethanol. The mouse microglial cell line BV-2 (Pahan et al., 2001) and the mouse macrophage cell line RAW264.7 (Nguyen and Benveniste, 2000) were grown in DMEM supplemented with 2 mM L-glutamine, 10% HI-FBS, and 2 mM sodium pyruvate. All experiments were performed in the presence of 10% HI-FBS. THP-1 (TIB-202) and RAW264.7 (TIB-71) cell lines were obtained from the American Type Cell Culture (Manassas, VA), and the BV-2 cell line was the kind gift of Dr. Michael McKinney (Mayo Clinic, Jacksonville, FL). Primary human monocyte-derived macrophages were derived from peripheral blood mononuclear cells (PBMCs) isolated from the blood of volunteers after obtaining informed consent from each individual. From each donor, 40 ml of heparinized blood was separated on Ficoll-Paque gradients (Pharmacia), and the mononuclear fraction was isolated. Macrophages were then enriched by plastic adherence. Briefly, PBMCs in RPMI supplemented with 20% HI-human serum (HI-HS) and 10% giant cell tumor conditioned medium (GCT-CM) (Igen International, Gaithersburg, MD) were plated at a density of 5×10^6 cells/ml in six-well plates. After 2 hr at 37°C, nonadherent cells were removed by stringent washing with complete RPMI media. Adherent monocytes were then differentiated in RPMI medium supplemented with 20% HI-HS and 10% GCT-CM for 5 d before use. The purity of the resulting cultures was 96%, as determined by staining for CD14, a macrophage/monocyte marker.

RNA isolation, riboprobes, and RNase protection assay. BV-2 and RAW264.7 cells were plated at 1×10^6 cells per 60 mm² dish (Costar, Cambridge, MA). After confluence, the medium was replaced, and cells were stimulated for the indicated times. THP-1 cells were plated at 4×10^6 cells per 60 mm² dish and stimulated immediately thereafter. Total cellular RNA was isolated at indicated times as described previously (Van Wagoner et al., 2000).

A pGEM-4Z vector containing a fragment of the human OSM cDNA corresponding to bp 1362–1660 (Malik et al., 1989) inserted at polylinker sites *Bam*HI–*Sac*I was linearized with *Eco*RI. *In vitro* transcription of this fragment with T7 RNA polymerase generates a 340 bp antisense RNase protection assay (RPA) probe. A pGEM-3Z vector containing a fragment of the mouse OSM (mOSM) cDNA, obtained from Dr. Kazuo Maruyama (DNAX Research Institute, Palo Alto, CA) (Yoshimura et al., 1996), corresponding to bp 777–1364 inserted at polylinker sites *Bam*HI–*Sac*I, was linearized with *Ava*I. *In vitro* transcription of this fragment with T7 RNA polymerase generates a 238 bp antisense RPA probe. A pAMP-1 vector containing a fragment of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA corresponding to bp 43–531 was linearized with *Not*I, and *in vitro* transcription of this fragment with T7 RNA polymerase generated a 290 bp antisense RPA probe. A pGEM-4Z vector containing a fragment of the mouse GAPDH cDNA corresponding to bp 223–434 inserted at polylinker sites *Eco*RI–*Kpn*I was linearized with *Eco*RI. *In vitro* transcription of this fragment with T7 RNA polymerase generated a 270 bp antisense RPA probe.

In vitro transcription of riboprobes was performed with the T7 *in vitro* transcription kit (Ambion, Austin, TX) in a final volume of 20 μ l containing 40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 500 mM ATP, CTP, and GTP, 10 mM DTT, 25 U of ribonuclease inhibitor, 12.5 μ M of [α -³²P] UTP (800 Ci/ml, 40 mCi/ml), 2 μ g of linearized DNA, and 10 U of T7 RNA polymerase at room temperature for 60 min, as described previously (Van Wagoner et al., 2000). The resulting radiolabeled transcripts were purified twice by phenol extraction and once by ethanol precipitation.

RPA was performed with the RPA kit according to the manufacturer's instructions, as described previously (Van Wagoner et al., 2000). Briefly,

20 μ g of total RNA was hybridized with appropriate riboprobes containing 30×10^3 cpm per probe per sample at 42°C overnight in 20 μ l of 40 mM PIPES, pH 6.4, 80% deionized formamide, 400 mM sodium acetate, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1:200 dilution in RNase digestion buffer, to yield 200 μ l/sample) at room temperature for 1 hr and then analyzed by 5% denaturing (8 M urea) PAGE. The protected fragments of human OSM (hOSM), hGAPDH, mOSM, and mGAPDH riboprobes were 298, 230, 223, and 212 bp in length, respectively. Quantification of the protected RNA fragments was performed by scanning with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for hOSM or mOSM mRNA were normalized to respective GAPDH mRNA levels for each experimental condition. GAPDH was chosen as a housekeeping gene because its levels are not affected by treatment with the described cytokines or prostaglandin.

OSM and PGE₂ ELISA. THP-1 cells were plated at 1×10^6 cells per well in six-well plates (Costar) in a total volume of 2 ml. Cells were stimulated with PGE₂, PGE₁, or norepinephrine for 2–24 hr. For coculture experiments, CRT-MG cells were plated at 0.5×10^6 cells per well in six-well plates and left to adhere overnight. The following day the media was changed, and six-well transwells, pore size 0.4 μ m (Costar), were added to each well. THP-1 cells were plated at 1×10^6 cells per well in the upper chamber of the transwells. Supernatants were collected at the indicated times and centrifuged for 15 sec at 14,000 rpm to precipitate cells. Cell-free supernatants were analyzed for the presence of OSM or PGE₂ according to the manufacturer's directions. Colorimetric measurements were performed using the Microplate reader 3550 (Bio-Rad, Hercules, CA). Cells were lysed in 100 μ l of lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton-100, 2 mM EDTA) by rotation for 1–2 hr at 4°C. Protein concentration was determined using Bio-Rad Protein Dye (Bio-Rad) according to the manufacturer's directions. OSM and PGE₂ concentrations were then normalized to the total protein concentration for each sample, and the data were expressed as picograms of OSM per milligram of total protein or picograms of PGE₂ per microgram of total protein.

cAMP ELISA. THP-1 cells were aliquoted at 1×10^6 cells per 1.5 ml Eppendorf tube in 1 ml total volume. Cells were stimulated with PGE₂ (0.001–10 μ M) for 5–150 min. At the indicated times, cells were centrifuged for 15 sec at 14,000 rpm. After the supernatants were aspirated, cells were lysed in 500 μ l of 0.1N HCl for 15 min and then centrifuged at 600 $\times$ g for 5 min to remove cell debris. The supernatant of the lysate was assayed for cAMP content according to the manufacturer's directions. The cAMP concentration was then normalized to the total protein concentration for each sample, and the data were expressed as picomoles of cAMP per milligram of total protein.

Plasmids. The pGL3 vector containing a 8.5 kb hOSM promoter (hOSMp8.5) (Ma et al., 1999) was a kind gift from Dr. Y. Ma (Department of Veterans Affairs Medical Center, Boise, ID). Digestion of hOSMp8.5 with *Pml*I and *Kpn*I, followed by ligation, yielded a 4.7 kb hOSM promoter construct (hOSMp4.7). The dominant-negative PKA construct (DN-PKA) (Clegg et al., 1987), containing a cAMP-unresponsive regulatory subunit of PKA under the control of the mouse metallothionein-1 promoter, was a kind gift from Dr. R. Johnson (University of Alabama at Birmingham, Birmingham, AL). Control empty vector for DN-PKA was obtained by excision of a *Pst*I-flanked fragment from the DN-PKA plasmid, followed by ligation.

Transient transfection and analysis. For transient transfection, 1 μ g of the 4.7 kb OSM promoter construct (hOSMp4.7) was cotransfected with 0.1 μ g of the pCMV- β -galactosidase construct into 4×10^5 BV-2 cells in six-well plates using the LipofectAMINE Plus method according to the manufacturer's directions (Invitrogen, Carlsbad, CA). The transfection mixture was further supplemented with 0.5 μ g of an expression vector for the DN-PKA as indicated, or with 0.5 μ g of its empty backbone vector as control. pGL3-Basic was used as a negative (background) control in all experiments. After 3 hr of transfection, cells were allowed to recover for 3 hr before treatment with PGE₂ (1 μ M) for 3 hr, which we have determined previously to be optimal for PGE₂-induced activation of the hOSMp4.7 construct (data not shown). During the recovery period, ZnSO₄ (100 μ M) was added to cells to induce the expression of the DN-PKA (Clegg et al., 1987). Cells were washed with PBS and lysed with 250 μ l of lysis buffer (25 mM trisphosphate, pH 7.8, 2 mM dithiothreitol, 2 mM diaminocyclohexane tetraacetic acid, 10% glycerol, and 1% Triton X-100). Extracts were assayed in triplicate for luciferase activity in a total volume of 130 μ l (30 μ l of cell extract, 20 mM Tricine, 0.1 mM EDTA, 1 mM MgCO₃, 2.67 mM MgSO₄, 33.3 mM dithiothreitol,

0.27 mM coenzyme A, 0.47 mM luciferin, and 0.53 mM ATP), and light intensity was measured using a luminometer (Promega, Madison, WI) as described previously (Nguyen and Benveniste, 2000). Luciferase activity was integrated over a 10 sec time period. Extracts were also assayed in triplicate for β -galactosidase enzyme activity as described previously (Nguyen and Benveniste, 2000). The luciferase activity of each sample was normalized to β -galactosidase activity to yield relative luciferase activity (RLA). Fold induction was calculated as the ratio of RLA between PGE₂ and medium-treated samples that were transfected with the same construct.

Statistical analysis. Levels of significance for comparisons between samples were determined using Student's *t* test distribution.

RESULTS

PGE₂ induces oncostatin-M expression in microglia, macrophages, and monocytes

Previous work in our laboratory indicated that astrocytes stimulated with IL-1 β secrete a soluble factor capable of inducing OSM synthesis in the monocytic cell line THP-1 (data not shown). This factor was not GM-CSF, one of the previously described OSM inducers (Ma et al., 1999). To establish its identity, we screened several chemokines, cytokines, and eicosanoids induced by IL-1 β treatment of astrocytes for their ability to upregulate OSM in THP-1 cells. Of the compounds we tested, only PGE₂ treatment of THP-1 cells resulted in a time- and dose-dependent induction of OSM expression. OSM mRNA was induced within 1 hr of PGE₂ treatment and returned to baseline levels by 3 hr, as determined by RPA (Fig. 1*A,B*). Optimal mRNA induction in THP-1 cells was attained with 0.1 μ M of PGE₂ (data not shown). Similar kinetics of OSM mRNA induction were noted in the EOC 20 and BV-2 murine microglial cell lines, as well as in the RAW264.7 murine macrophage cell line (data not shown). In murine microglial (BV-2) and macrophage (RAW264.7) cell lines, PGE₂ at 1–10 μ M induced OSM mRNA expression, but a lower concentration of 0.1 μ M did not (Fig. 1*C*) (data not shown).

Accordingly, the level of OSM protein in the supernatants of THP-1 cells increased steadily after PGE₂ addition, peaking at ~21 hr as determined by OSM ELISA (Fig. 2*A*). Although this experiment was extended to 72 hr, no further increase in OSM levels was detected (data not shown), indicating that maximal OSM protein production was attained by 21 hr. Optimal OSM production in THP-1 cells was observed after treatment with 0.01 μ M PGE₂ (Fig. 2*B*). Primary human monocyte-derived macrophages also produced OSM in response to PGE₂, exhibiting a response comparable to that of THP-1 cells (Fig. 2*C*). Taken together, these data establish PGE₂ as a novel inducer of OSM expression in both human and murine cells of monocytic lineage.

PGE₂ treatment elevates intracellular cAMP levels

PGE₂ signals through transmembrane G-protein-coupled receptors, of which four types (EP1–EP4) have been identified so far (Narumiya and FitzGerald, 2001). Depending on which of the four receptor types it signals through, PGE₂ can lead to an elevation or depression of intracellular cAMP levels or to an increase in intracellular Ca²⁺ (Narumiya et al., 1999). To determine which of these events takes place in our system, we monitored the levels of intracellular cAMP and Ca²⁺ after PGE₂ treatment of THP-1 human monocytic cells. PGE₂ elicited a large and sustained increase in intracellular cAMP concentration, as determined by cAMP ELISA (Fig. 3*A*). Maximal cAMP levels were observed after 30 min of PGE₂ stimulation and returned to basal levels by 150 min. The kinetics of this response agrees well with PGE₂ induction of OSM mRNA, as does the dose–response curve; the optimal cAMP increase was seen after treatment with

Figure 1. PGE₂ induces OSM mRNA expression in cells of monocytic lineage. THP-1 monocytic cells (4×10^6), plated in 60 mm dishes, were stimulated with PGE₂ (0.1 μ M) for 1–3 hr. Total RNA was extracted and analyzed by RPA for OSM and GAPDH mRNA (*A*). Quantification of the RPA blot is shown in *B*, with OSM mRNA expression normalized to GAPDH levels, and plotted as fold induction over control. Results are representative of at least three experiments. BV-2 murine microglial cells and RAW264.7 murine macrophage cells, plated in 60 mm dishes (4×10^6), were stimulated with PGE₂ (1–10 μ M) for 1 hr. Total RNA was extracted and analyzed by RPA for OSM and GAPDH mRNA expression. Results are representative of at least two independent experiments (*C*).

0.1 μ M PGE₂ (Fig. 3*B*). Intracellular Ca²⁺ levels, on the other hand, were not significantly affected by PGE₂ treatment (data not shown). Therefore, in THP-1 cells, PGE₂ treatment leads to an increase in intracellular cAMP levels.

Other agents that elevate cAMP also induce OSM

Given that PGE₂ induces both intracellular cAMP elevation and OSM expression, we hypothesized that cAMP was the secondary

Figure 2. Kinetics and dose-response of PGE₂-induced OSM protein expression. THP-1 cells, plated in six-well plates (2×10^6 /2 ml), were treated for 3–24 hr with PGE₂ (0.1 μM), and OSM concentrations in the supernatants were determined by ELISA (A). OSM concentrations were normalized to total protein levels. Data shown are the mean $\pm$ SD for three experiments. THP-1 cells (B) or primary human monocyte-derived macrophages (C) were plated in six-well plates and stimulated with PGE₂ (0.001–10 μM) for 24 hr, and OSM concentrations were determined as described above. Data shown are the mean $\pm$ SD for three experiments.

messenger through which PGE₂ induced OSM expression. In this scenario, cAMP elevation would be both necessary and sufficient for OSM induction. To demonstrate that, we treated THP-1 cells and primary human macrophages with other agents that elevate intracellular cAMP. Norepinephrine and prostaglandin E₁ (PGE₁) mimicked the effect of PGE₂ on OSM induction in

Figure 3. PGE₂ elevates cAMP levels in THP-1 cells. THP-1 cells (0.5×10^6 /ml) were treated with PGE₂ (0.1 μM) for 5–150 min. Cells were then lysed, and intracellular cAMP concentrations were determined by ELISA (A). THP-1 cells were treated for 30 min with PGE₂ (0.001–10 μM), and intracellular cAMP levels were determined by ELISA (B). Data shown are the mean $\pm$ SD of three experiments.

primary human macrophages (Fig. 4A). Similar effects were seen in THP-1 cells (data not shown). CTX, which activates the G_s-proteins by ADP-ribosylation of their α subunits (Gill and Meren, 1978), leading to activation of adenylate cyclase and subsequent cAMP elevation, potentially induced OSM synthesis in THP-1 cells (Fig. 4B). FSK, an activator of adenylate cyclase (Seamon et al., 1981), also induced OSM (Fig. 4B). Finally, dbcAMP, a synthetic, cell-permeable analog of cAMP (Ahn et al., 1969), induced OSM production in THP-1 cells (Fig. 4B). Taken together, these data confirm that elevation of intracellular cAMP in macrophages–monocytes is sufficient to induce OSM expression.

Inhibitors of cAMP production and PKA activation block PGE₂-mediated OSM induction

To test whether cAMP elevation is also necessary for OSM induction by PGE₂, we used pharmacological inhibitors of the cAMP cascade. Treatment of THP-1 cells with DDA, an inhibitor of adenylate cyclase (Zenser and Wannemacher, 1976), led to a partial, but significant, decrease in PGE₂-induced OSM mRNA and protein (Fig. 5A–C). The inhibition was not complete (~40–45%), possibly because of the fact that DDA is a reversible inhibitor.

Another inhibitor of the cAMP signaling cascade showed a

Figure 4. Agents that increase intracellular cAMP levels induce OSM. Primary human monocyte-derived macrophages were plated in six-well plates and treated with PGE₁ or norepinephrine (NE) (0.01–10 μM) for 24 hr. OSM concentrations in the supernatants were measured by ELISA and normalized to total protein. Data shown are the mean ± SD of three experiments (A). THP-1 cells plated in six-well plates were stimulated with the indicated doses of cholera toxin (CTX), forskolin (FSK), or dibutyryl-cAMP (dbcAMP) for 24 hr. OSM concentrations in the supernatants were determined by ELISA and normalized to total protein. Shown are the mean ± SD of three experiments (B).

more pronounced effect. H-89, an inhibitor of PKA (Chijiwa et al., 1990), completely blocked PGE₂-mediated OSM expression in THP-1 cells. The inhibitory effect was observed at both the mRNA and protein levels (Fig. 6A–C) in a dose-dependent manner. Although H-89 can inhibit kinases other than PKA, in the concentration range that we used, it is considered specific for PKA (Lee and Linstedt, 2000). The solvent of H-89, DMSO, was used as a control in corresponding volumes but had no effect on PGE₂-induced OSM mRNA expression (Fig. 6B). These experiments indicate that cAMP is both sufficient and necessary for PGE₂-induced OSM expression in cells of monocytic origin.

The inactive PKA mutant abrogates PGE₂-mediated induction of OSM promoter activity

Previous results with the H-89 inhibitor suggested a crucial role for PKA in OSM induction by PGE₂. To better understand the events leading to OSM activation, including the role of PKA, we generated a construct of the luciferase gene under the control of a 4.7 kb hOSM promoter (hOSMp4.7), as described in Materials and Methods. We were not successful in our attempts to introduce hOSMp4.7 into THP-1 cells, because these cells were not

Figure 5. PGE₂-induced OSM expression is partially inhibited by the adenylate cyclase inhibitor DDA. THP-1 cells plated in six-well plates were pretreated for 30 min with DDA (1–2 mM), before stimulation with PGE₂ (0.1 μM). After 6 hr, OSM concentrations in the supernatants were determined by ELISA. The OSM concentration in the sample without the inhibitor was set to 100%, and other concentrations represent a percentage fraction of this maximal induction. Data shown are the mean ± SD of three experiments. The difference between PGE₂-treated and inhibitor-treated samples was significant as determined by the Student's *t* test. **p* < 0.05; *n* = 3 (A). THP-1 cells plated in 60 mm dishes were pretreated with DDA (2 mM) for 30 min and then treated with medium or PGE₂ (0.1 μM) for 1 hr. Total RNA was extracted and RPA analysis was performed for OSM and GAPDH mRNA (B). Quantification of the RPA blot is shown in C. Data shown are representative of three independent experiments.

amenable to transfection (data not shown). However, transient transfection of the hOSMp4.7 construct in BV-2 murine microglial cells produced a suitable system for the study of OSM promoter activity after stimulation with PGE₂. In this system, treatment of transiently transfected BV-2 cells with PGE₂ activates the OSM promoter, resulting in approximately a twofold

Figure 6. Inhibition of protein kinase A abrogates PGE₂-induced OSM expression. THP-1 cells plated in six-well plates were pretreated with H-89 (5–20 μM) for 1 hr before stimulation with PGE₂ (0.1 μM) for 24 hr. OSM concentrations in the supernatants were measured by ELISA and normalized to total protein per well. Data shown are the mean ± SD of three experiments. The difference between PGE₂-treated and inhibitor-treated samples was significant as determined by the Student's *t* test. **p* < 0.05; *n* = 3 (*A*). For RPA analysis of OSM and GAPDH mRNA, THP-1 cells plated in 60 mm dishes were pretreated with H-89 (5–10 μM) or the corresponding volume of solvent (DMSO) for 1 hr before stimulation with PGE₂ (0.1 μM) for 1 hr (*B*). Quantification of the RPA blot is shown in *C*. Data shown are representative of three experiments.

induction of relative luciferase activity as compared with medium-treated controls (Fig. 7). Although this level of fold induction was not high, it enabled us to investigate the effect of the DN-PKA mutant (Clegg et al., 1987) on OSM promoter activity elicited by PGE₂. The DN-PKA construct contains a cAMP-unresponsive regulatory subunit of PKA under the control of a mouse metallothionein-1 promoter that is activated by the addition of ZnSO₄ to the media (Clegg et al., 1987). The

Figure 7. Dominant-negative PKA abrogates PGE₂-induced activation of OSM promoter activation. BV-2 cells were transiently transfected with a 4.7 kb OSM promoter (1 μg) plus empty vector (0.5 μg) or expression vector for a dominant-negative regulatory subunit of PKA (DN-PKA) (0.5 μg). In addition, all cells were cotransfected with a β-galactosidase vector (0.1 μg). Cells were treated with ZnSO₄ (0.1 mM) for 3 hr before stimulation with PGE₂ (1 μM) for 3 hr. Cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity as described in Materials and Methods. Fold induction represents a ratio of normalized luciferase activity of PGE₂-treated sample over untreated control. Results are representative of at least three independent experiments performed in triplicate.

DN-PKA construct or its empty vector control were transiently cotransfected into BV-2 cells with the hOSMp4.7 construct. ZnSO₄ was subsequently added to stimulate the expression of DN-PKA. We confirmed that ZnSO₄ does not affect luciferase induction by PGE₂ and that the empty vector control also has no inhibitory effect on hOSMp4.7 (Fig. 7). However, the DN-PKA activated by ZnSO₄ completely abrogated PGE₂ induction of OSM promoter activity (Fig. 7). These data unequivocally demonstrate that PKA is necessary for OSM induction by PGE₂.

The role of PGE₂ in mediating OSM induction resulting from the inflammatory stimulation of astrocyte–monocyte cocultures

In the experiments thus far, to demonstrate the induction of OSM by PGE₂, we relied on the addition of exogenous PGE₂. However, to establish PGE₂ as a physiologically relevant OSM inducer, we sought an endogenous source of PGE₂. Our earliest experiments suggested that astrocytes could serve this purpose, because their ability to produce PGE₂ after stimulation with IL-1β or TNF-α is well known (Hartung et al., 1989; Blom et al., 1997; Janabi et al., 1999; Pistritto et al., 1999; Molina-Holgado et al., 2000). A coculture model of astrocytes and monocytes was thus established using the CRT-MG human astrogloma cell line and the THP-1 monocytic cell line. We hypothesized that in these cocultures, IL-1β treatment would induce PGE₂ synthesis, which would in turn lead to the expression of OSM. When cocultures of CRT-MG astrogloma cells and THP-1 monocytic cells were treated with IL-1β for 24 hr, the levels of PGE₂ in the supernatants increased, as did the levels of OSM, according to our prediction (Fig. 8*A*). Of note, IL-1β treatment of each cell type alone did not stimulate OSM expression (data not shown).

To demonstrate that PGE₂ is responsible for the induction of OSM in the cocultures, PGE₂ production was inhibited using a nonselective cyclooxygenase inhibitor, indomethacin (Rozic et al., 2001). Pretreatment of the cocultures with indomethacin (1 μM) for 30 min before IL-1β treatment inhibited the induction of OSM (Fig. 8*B*). This suggested a critical role for cyclooxygenase

Figure 8. Endogenously produced PGE₂ mediates OSM induction in astrocyte-monocyte cocultures. THP-1 cells (1×10^6) and CRT-MG astrogloma cells (5×10^5) were plated in the upper and lower chambers, respectively, of six-well transwell plates and stimulated with IL-1β (0.001–10 ng/ml). After 24 hr, the supernatants were harvested, and PGE₂ and OSM concentrations were determined using respective ELISA kits. OSM concentrations, normalized to total cell protein, are plotted against the left y-axis, whereas similarly normalized PGE₂ concentrations are plotted against the right y-axis. Data shown are the mean $\pm$ SD of three

product(s) in the induction of OSM. To demonstrate that PGE₂ specifically mediates OSM production in the coculture model, we performed supernatant transfer experiments. Supernatants from astrocytes treated with IL-1β or TNF-α for 24 hr were harvested and transferred to THP-1 monocytic cells, where they induced OSM expression (Fig. 8C). When these supernatants were specifically depleted of PGE₂ before their transfer to THP-1 cells, using anti-PGE₂-coated Sepharose beads, their ability to upregulate OSM expression was lost (Fig. 8C). Taken together, these data unequivocally implicate PGE₂ as an endogenous OSM inducer in the coculture of astrocytes and monocytes and suggest it as a likely OSM inducer in other, more physiological settings *in vivo*.

To confirm that OSM in the cocultures is of monocytic origin, astrocytes and monocytes were cocultured in upper and lower transwell chambers, respectively, and incubated in the absence or presence of IL-1β for 12–24 hr, and then RNA was isolated from each cell population. OSM mRNA was detected only in the monocytic cells, but not in astrocytes, in the samples treated with IL-1β (Fig. 8D). These results confirm that monocytes are indeed the source of OSM in the cocultures. We have attempted similar experiments using primary cultures of murine microglia and astrocytes. These experiments are complicated by the fact that primary murine microglia express high basal levels of OSM mRNA, likely because of activation during the purification and plating procedures. Nonetheless, OSM mRNA expression was enhanced by approximately twofold in the microglial population after IL-1β stimulation, whereas primary astrocytes did not express OSM mRNA (data not shown).

DISCUSSION

OSM synthesis is induced by various physiological, pharmacological, and viral stimuli. Physiological inducers of OSM expression include members of the IL-3 cytokine family (IL-3 and GM-CSF), as well as hCG. Only for IL-3 and GM-CSF has the mechanism of OSM induction been described (Ma et al., 1999). These cytokines have been shown to activate STAT-5, which binds to STAT-responsive elements in the OSM promoter, leading to OSM transcription. In this study, we describe an entirely novel class of OSM inducers, represented by PGE₂ (Figs. 1, 2), that use a cAMP signaling pathway to elicit OSM expression.

PGE₂ signals through transmembrane G-protein-coupled receptors, of which four types have been identified to date (Narumiya and FitzGerald, 2001). Stimulation of EP1 elevates intracel-

← experiments (A). THP-1 cells and CRT-MG cells plated as above in six-well transwells were pretreated with indomethacin (1 μM) for 30 min before treatment with IL-1β (0.1–1 ng/ml) for 24 hr. OSM concentrations in the supernatants were determined using ELISA. Data shown are the mean $\pm$ SD of three experiments. The difference between IL-1β-treated and indomethacin-treated samples was significant as determined by the Student's *t* test. **p* < 0.05; *n* = 3 (B). CRT-MG cells (1×10^5) were plated in six-well plates and stimulated with IL-1β (0.1 ng/ml) or TNF-α (50 ng/ml) for 24 hr. Aliquots (1 ml) of the supernatants were incubated with either anti-PGE₂-coated Sepharose beads or plain Sepharose beads for 1 hr at 37°C before being added to THP-1 cells (1×10^6) for 24 hr. The concentration of OSM in the THP-1 supernatants was determined using ELISA. Data shown are the mean $\pm$ SD of three experiments. The difference between samples incubated with Sepharose beads and anti-PGE₂-coated beads was significant as determined by the Student's *t* test. **p* < 0.05; *n* = 3 (C). Astrocytes (2×10^5) and monocytes (3×10^5) were cocultured in transwell plates and stimulated with IL-1β (0.1 ng/ml) for 12–24 hr. At indicated times, RNA was harvested and analyzed by RPA for OSM and GAPDH mRNA. Data shown are representative of two independent experiments (D).

lular Ca²⁺ (Asboth et al., 1996). Stimulation of EP2 or EP4 elevates intracellular cAMP, because these receptors are coupled to G_s-proteins, and thus activate adenylate cyclase (Fedyk and Phipps, 1996). On the other hand, EP3 stimulation decreases intracellular cAMP because this receptor is coupled to inhibitory G-proteins (Namba et al., 1993). We have shown that PGE₂ treatment of monocytic cells leads to cAMP elevation (Fig. 3), suggesting that EP2 and/or EP4 transduce the PGE₂ signal in macrophages and microglia, as reported previously (Caggiano and Kraig, 1999; Patrizio et al., 2000). The cAMP generated by adenylate cyclase activates the cAMP-dependent protein kinase (PKA) by binding to its regulatory units and releasing the catalytic subunits (Knight and Fordham, 1975). The catalytic units of PKA subsequently diffuse to the nucleus (Montminy, 1997), where they phosphorylate corresponding transcription factors that result in OSM expression.

The central role of PKA activation in OSM induction is underscored by our finding that agents other than PGE₂ that activate PKA, such as norepinephrine, PGE₁, CTX, forskolin, or dbcAMP, are also capable of inducing OSM expression (Fig. 4). Furthermore, the PKA inhibitor H-89, in a dose-dependent manner, abrogates OSM expression induced by PGE₂ (Fig. 6). Although there are reports that H-89 may interfere, at higher concentrations, with protein transport (Lee and Linstedt, 2000), our RPA results indicate that H-89 directly blocks PGE₂-induced OSM mRNA expression, rather than the transport of translated OSM protein. That this action of H-89 is caused by inhibition of PKA, rather than another kinase, is further indicated by our finding that a dominant-negative mutant of the PKA regulatory subunit (Clegg et al., 1987) completely abolishes OSM promoter activity induced by PGE₂ stimulation (Fig. 7). Because PKA activation plays such a crucial role in OSM expression, additional OSM inducers may yet be found among other physiological PKA activators, such as vasoactive intestinal peptide (Guerrero et al., 1984), histamine (Shayo et al., 1997), or substance P (Mitsuhashi et al., 1992).

Our data indicate that the signaling pathway used by PGE₂ to induce OSM synthesis in monocytic cells is a typical cAMP signaling cascade, where PGE₂ treatment results in PKA activation and ultimately OSM expression. For the time being, the molecular link between PKA activation and the onset of OSM transcription remains unknown. It seems likely that PKA-activated transcription factors, such as cAMP-responsive element-binding protein (CREB) (Montminy and Bilezikjian, 1987), play a role in this process, because there are several potential CREB binding sites in the OSM promoter, as predicted by MatInspector software (Quandt et al., 1995). Determining which transcription factors are involved, and what region of OSM promoter they bind to, will be the subject of future investigation.

Our discovery of PGE₂ as a novel inducer of OSM prompted us to test its OSM-inducing capacity under more physiological conditions. To do so, we used an *in vitro* coculture of astrocytes and monocytes as a system in which PGE₂ would be endogenously produced, rather than added exogenously. The astrocyte–monocyte coculture presented itself as a suitable system for several reasons. First, astrocyte–monocyte cocultures are an established *in vitro* model and have been used extensively in the study of HIV-1-associated dementia (Genis et al., 1992; Fiala et al., 1996; Pereira et al., 2001) and chemokine expression in the CNS (Andjelkovic et al., 2000). Second, the capacity of astrocytes to produce PGE₂ after stimulation with IL-1β or TNF-α has been well documented (Janabi et al., 1999; Pistrutto et al., 1999;

Figure 9. Two-step model of OSM production. In the first step, IL-1β or TNF-α act on astrocytes to release PGE₂ as a diffusible inducer of OSM synthesis. In the second step, this inducer stimulates macrophages or microglia to produce OSM.

Molina-Holgado et al., 2000). Third, although astrocytes produce copious amounts of PGE₂, we have found that they do not express OSM in response to IL-1β or PGE₂ stimulation (Fig. 8D) (data not shown). Finally, the astrocytic and monocytic cell lines that we used require identical media formulations, which allowed us to coculture them under the conditions optimal for both cell types. In this coculture of CRT-MG astrogloma cells and THP-1 monocytic cells, we demonstrated that IL-1β stimulation induces a dose-dependent increase in OSM synthesis, which paralleled the increase in endogenous PGE₂ levels (Fig. 8). Furthermore, if IL-1β- or TNF-α-treated astrocyte supernatants were specifically depleted of PGE₂, they lost their ability to induce OSM.

On the basis of these results, we derived a two-step model of OSM synthesis that operates in our coculture model and perhaps also in the CNS (Fig. 9). In the first step, inflammatory mediators such as IL-1β or TNF-α stimulate PGE₂ production by astrocytes. In the second step, this PGE₂ acts on nearby microglia or macrophages to induce OSM synthesis. This model need not be confined only to astrocytes and microglia. The first step may also apply to other sources of PGE₂ in the CNS such as endothelial cells (Yamagata et al., 2001) or neurons (Rettori et al., 1992; Nogawa et al., 1997), as our preliminary *in vitro* data would indicate (data not shown). Unlike the first step, the second step of the model appears to be restricted to the cells of monocytic origin (monocytes, macrophages, and microglia), because a number of other cell types reported to produce OSM (T-cells, neutrophils, endothelial cells, and neuroblastoma cells) failed to express OSM in response to PGE₂ (data not shown). Because most of these cell types express PGE₂ receptors, it seems likely that restricted OSM expression in monocytes, macrophages, and microglia is caused by the cell-specific distribution of appropriate transcription factors. *In vivo* CNS expression of OSM has been described recently (Ruprecht et al., 2001). OSM immunoreactivity was detected in multiple sclerosis (MS) lesions, and staining was most prominent in microglial cells and hypertrophic astrocytes. No OSM expression was observed in normal brain and noninflammatory neurological disease. Hallmarks of MS include elevated CNS levels of IL-1β, TNF-α, and PGE₂ (for review, see Brosnan and Raine, 1996); thus, all the stimulatory components are present to elicit OSM expression. These results support our *in vitro* observations

of OSM expression by microglia–macrophages. We did not detect astrocytic production of OSM in the coculture experiments in the presence of IL-1 β (Fig. 8D). Although *in vivo* expression of OSM by astrocytes has been observed, our *in vitro* system may lack the inducing stimuli necessary for astrocytic expression of OSM.

The two-step model of OSM induction may also extend beyond the CNS, thus offering an explanation for some of the previous reports of elevated OSM levels in inflammatory pathologies. In rheumatoid arthritis, for example, OSM levels correlate with disease severity (Okamoto et al., 1997; Manicourt et al., 2000), whereas in atherosclerosis, OSM and TNF- α colocalize in atherosclerotic plaques (Vasse et al., 1999; Barillari et al., 2001). OSM is also elevated in some forms of breast cancer (Crichton et al., 1996), a condition with documented upregulation of COX-2 (Soslow et al., 2000), as well as in hepatic cirrhosis (Levy et al., 2000), systemic sclerosis (Hasegawa et al., 1998), and endotoxin-induced renal disease (Baumann et al., 2000).

The proposed IL-1 β –PGE₂–OSM cascade incorporates OSM in the inflammatory sequence of events. However, the subsequent role of OSM in this milieu remains a subject of debate. Like its related cytokine IL-6, OSM is known to exhibit both pro- and anti-inflammatory properties, depending on the context and disease model under study (for review, see Van Wagoner and Benveniste, 1999; Wahl and Wallace, 2001). In a model of HIV-1-associated neurodegeneration, OSM induced neuronal damage (Ensoli et al., 1999), whereas in the murine model of experimental allergic encephalomyelitis, administration of OSM suppressed the inflammatory response and tissue damage in the CNS that is characteristic of this model (Wallace et al., 1999). This anti-inflammatory effect of OSM may be caused by its ability to inhibit TNF- α production (Wallace et al., 1999). However, in endothelial cells, OSM exerts pro-inflammatory effects via the induction of adhesion molecules and chemokines (Modur et al., 1997). Thus, the ultimate response to OSM, either pro- or anti-inflammatory, may be cell-type specific or tissue specific. Clearly, more needs to be done to determine the exact role of OSM in CNS inflammation. For this reason, understanding the regulation of OSM expression is essential. In this study, we contribute to answering this question by presenting the data that outline a new mechanism of OSM production that may be operational within the CNS as well as in the rest of the body. Further work will be necessary to elucidate events that follow PKA activation and lead to OSM transcription and to address the issue of cell-type-specific expression of OSM.

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