Dexamethasone Enhances ATP-Induced Inflammatory Responses in Endothelial Cells

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Received June 24, 2010; accepted September 2, 2010

ABSTRACT

The purinergic nucleotide ATP is released from stressed cells and is implicated in vascular inflammation. Glucocorticoids are essential to stress responses and are used therapeutically, yet little information is available that describes the effects of glucocorticoids on ATP-induced inflammation. In a human microvascular endothelial cell line, extracellular ATP-induced interleukin (IL)-6 secretion in a dose- and time-dependent manner. When cells were pretreated with dexamethasone, a prototypic glucocorticoid, ATP-induced IL-6 production was enhanced in a time- and dose-dependent manner. Mifepristone, a glucocorticoid receptor antagonist, blocked these effects. ATP-induced IL-6 release was significantly inhibited by a phospholipase C inhibitor [1-[6-[(17 β)-3-methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122)] ($63.2 \pm 3\%$, $p < 0.001$) and abolished by a p38 mitogen-activated protein kinase inhibitor [4-(4-fluorophenyl)-2-

(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB 203580)] ($88 \pm 1\%$, $p < 0.001$). Cells treated with dexamethasone induced mRNA expression of the purinergic P2Y₂ receptor (P2Y₂R) 1.8- $\pm$ 0.1-fold and, when stimulated with ATP, enhanced Ca²⁺ release and augmented IL-6 mRNA expression. Silencing of the P2Y₂R by its small interfering RNA decreased ATP-induced IL-6 production by $81 \pm 1\%$ ($p < 0.001$). Dexamethasone enhanced the transcription rate of P2Y₂R mRNA and induced a dose-related increase in the activity of the P2Y₂R promoter. Furthermore, dexamethasone-enhanced ATP induction of adhesion molecule transcription and augmented the release of IL-8. Dexamethasone leads to an unanticipated enhancement of endothelial inflammatory mediator production by extracellular ATP via a P2Y₂R-dependent mechanism. These data define a novel positive feedback loop of glucocorticoids and ATP-induced endothelial inflammation.

Introduction

Microvascular endothelial cells play a pivotal role in inflammation by the release of inflammatory mediators and

the expression of adhesion molecules that recruit inflammatory cells from blood to tissues (Swerlick and Lawley, 1993; Krishnaswamy et al., 1999; Pober et al., 2009). ATP is a multifunctional nucleotide that serves as an energy source, a component of RNA, and a substrate for intracellular signaling. An additional dimension to the actions of ATP includes its role as an extracellular mediator when released by excitable (i.e., neuron) and nonexcitable (i.e., endothelium, epithelium, myeloid) tissues (Abbracchio et al., 2006; Chen et al., 2006). ATP release may occur constitutively (Schwiebert et al., 2002) or after cell stimulation by depolarization, shear stress, or hypoxia (Rich et al., 2003; Abbracchio et al., 2006). Depending on the cell type, receptor agonists that promote ATP release include bradykinin, ADP, bacterial components,

This work was supported by the Intramural Research Program of the National Institutes of Health Clinical Center and the National Institute of Diabetes and Digestive and Kidney Diseases.

Presented previously, in part, Ding Y, Gao Z-G, and Suffredini AF (2009) Dexamethasone (DEX) upregulates the purinergic receptor P2Y₂ and enhances adenosine triphosphate (ATP)-induced IL-6 production in endothelium, at the American Thoracic Society annual conference; 2009 May 15-20; San Diego, CA. American Thoracic Society, New York, NY. (*Am J Respir Crit Care Med* 179:A2344).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.110.171975.

ABBREVIATIONS: P2, purinergic receptor 2; RU486, 11 β -(4-dimethylamino)phenyl-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one, mifepristone; U73122, 1-[6-[(17 β)-3-methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; PD 98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SP 600125, 1,9-pyrazoloanthrone, anthrapyrazolone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; IL, interleukin; TNF α , tumor necrosis factor α ; IL-1ra, IL-1 receptor antagonist; MCP-1, monocyte chemotactic factor; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; siRNA, small interfering RNA; PLC, phospholipase C; MAPK, mitogen-associated protein kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; [Ca²⁺]_i, intracellular calcium; JNK, c-Jun N-terminal kinase; TNFAIP3, TNF α -induced protein 3; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1; SELE, E-selectin; DEX, dexamethasone.

thrombin, and T-cell receptor activation (Di Virgilio et al., 2001a; Abbracchio et al., 2006; Bours et al., 2006). In addition, extracellular ATP release may occur after tissue damage and necrosis, and it represents a danger-associated molecular pattern molecule that can initiate inflammatory responses and activate both innate and adaptive immunity (Di Virgilio et al., 2001a; Abbracchio et al., 2006; Erlinge and Burnstock, 2008; Rao and Pober, 2008; Yu and Finlay, 2008). Understanding mechanisms that modulate ATP effects on target cells may have therapeutic implications.

Extracellular ATP may act in an autocrine or paracrine manner and may activate the P2 family of purinergic receptors expressed on many different tissues: P2X receptors are ligand-gated ion channel receptors, and P2Y receptors are G protein-coupled receptors that elicit diverse responses (Abbracchio et al., 2006; Erlinge and Burnstock, 2008). After receptor binding, extracellular ATP may initiate and modulate inflammation in several ways: activating cells (e.g., endothelium, leukocytes), inducing cytokine and chemokine release, enhancing expression of adhesion molecules, and facilitating chemotaxis (Di Virgilio et al., 2001b; Bours et al., 2006; Yu and Finlay, 2008). Termination of these responses is mediated by ectonucleotidases that are present in the circulation and on cell surfaces (Bours et al., 2006). Extracellular ATP has thus been implicated in vascular inflammation, atherosclerosis, and angiogenesis.

The development of shock and organ failure from severe infections or hemorrhage is associated with a decrease in tissue ATP levels (Chaudry et al., 1976; Brealey et al., 2002). Plasma ATP levels fall with shock (Jabs et al., 1979; Seekamp et al., 1999), but these measurements may be confounded by failure to account for ATP release from blood elements and extracellular ATP catabolism (Gorman et al., 2007). Newer analytical methods show that ATP levels in some pericellular environments may be in the range of several hundred micromolar (Abbracchio et al., 2006; Bours et al., 2006; Pellegatti et al., 2008).

Little information is available regarding the interactions of ATP and glucocorticoids on cell inflammatory responses. The release of stress hormones (e.g., cortisol, catecholamines) during injury or serious infections may alter the subsequent host responses to inflammatory stimuli (Barber et al., 1993; van der Poll et al., 1996). Although glucocorticoids are used to treat diverse inflammatory conditions, when present in excess they are associated with an increase in cardiovascular risk factors (i.e., hypertension, glucose intolerance, obesity, and dyslipidemia) and progression of atherosclerotic cardiovascular disease (Walker, 2007). Low doses of corticosteroids have become an important adjunctive therapy in the treatment of septic shock (Minnecci et al., 2009). Thus, endogenous and administered glucocorticoids have the potential to affect a myriad number of host inflammatory responses. To assess the potential interaction of glucocorticoids with inflammatory molecules released by cell activation or injury, we studied the interaction of a pure glucocorticoid agonist, dexamethasone, and ATP on endothelial cell-associated inflammatory responses.

Materials and Methods

Media and Cell Lines. The human dermal microvascular endothelial cell line-1 (HMEC-1; kindly provided by F. J. Candal, Centers

for Disease Control and Prevention, Atlanta, GA) immortalized by simian virus 40 transformation was cultured with MCDB 131 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), epidermal growth factor (10 ng/ml; BD Biosciences, San Jose, CA), hydrocortisone (1 μ g/ml; Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin/100 μ g/ml streptomycin (Invitrogen), and L-glutamine (2 mM; Invitrogen) in a humidified atmosphere at 37°C with 5% CO₂. Hydrocortisone was removed 24 h before the initiation of the experiments. Immortalized EA.hy926 endothelial cells (American Type Culture Collection, Manassas, VA), derived from the fusion of primary human umbilical vein endothelial cells with A549 lung carcinoma cells, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Reagents. ATP, dexamethasone, mifepristone (RU486), U73122, Gö6976, PD 98059, SB 203580, SP 600125, LY294002, suramin sodium salt, and actinomycin D were purchased from Sigma-Aldrich. Dexamethasone, RU486, U73122, Gö6976, PD 98059, SB 203580, and SP 600125 were dissolved in DMSO (Sigma-Aldrich). The final concentrations of DMSO in the culture medium were less than 0.1%, and these amounts were also included in the corresponding controls. Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4, ultrapure) was purchased from List Biological Laboratories Inc. (Campbell, CA).

Cytokine Measurements. HMEC-1 cells were cultured in 24-well plates (10⁵ cells/ml in each well) and stimulated under different conditions. The cell-free supernatant fractions were collected, and IL-6 was measured in duplicate by immunoassay (Quantikine Human IL-6 Immunoassay Kit; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. A multiplex assay for simultaneous measurement of nine secreted mediators (granulocyte colony-stimulating factor, IL-1 β , IL-6, IL-8, IL-10, TNF α , IL-1ra, MCP-1, and macrophage inflammatory protein-1 α) was performed using the Fluorokine MAP Human Base Kit A (R&D Systems).

Measurement of Intracellular Ca²⁺ Concentration in HMEC-1 Cells. Cells were seeded (10⁵ cells/ml) in 100 μ l of medium in 96-well plates for 24 h and then treated under different conditions, and intracellular calcium was measured (fluorometric imaging plate reader calcium assay kit; Molecular Devices, Sunnyvale, CA). Cells were loaded with 50 μ l of dye with probenecid in each well and were incubated for 45 min at room temperature. The compound plate was prepared with dilutions of various compounds in Hanks' buffer at pH 7.2. Samples were run in duplicate with a Flexstation I (Molecular Devices) at room temperature. Cell fluorescence (excitation, 485 nm; emission, 525 nm) was monitored after exposure to a compound. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.

RNA Isolation and Quantitative Real-Time PCR. Total RNA was extracted from HMEC-1 cells using the RNeasy Total RNA Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Total RNA (2 μ g) was reverse-transcribed with a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was amplified by PCR using the TaqMan probe for IL-6 and P2Y₂R. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control. Quantitative real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR condition was denaturing at 95°C for 15 s and annealing at 60°C for 1 min with 40 cycles. Signals were analyzed by the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All primers and reagents for PCR were purchased from Applied Biosystems.

RT-PCR Array. Eighty-four genes associated with endothelial cell function and five housekeeping genes were assayed using the Endothelial Cell Biology RT² Profiler PCR Array (SABiosciences, Frederick, MD). Total RNA was extracted as described above. RNA quality was assessed by UV spectrometry and electrophoresis on a denaturing agarose gel. The RNA was reverse-transcribed using the RT² First Strand Kit, using the same total amount of RNA for each reaction. The reverse-transcribed RNA was mixed with RT² qPCR

Master Mix and RT² SYBER Green Mix (Applied Biosystems) and applied to 384-well plates that were predisposed with gene-specific primer sets using the Biomek 2000 (Beckman Coulter, Fullerton, CA). A two-step cycling program was used (one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min). Data analysis was performed using the RT² Profiler PCR Array Data Analysis Template v3.0 (SABiosciences), using the mean values from at least three independent experiments. Significant differences between the treatment and the control values were determined by a two-tailed Student's *t* test with a nominal *p* value of <0.05 considered significant.

RNA Interference-Mediated Down-Regulation of P2Y₂R. The HMEC-1 cells were grown in 24-well plates at 30 to 50% confluence overnight and then transfected with 75 pmol of siRNA for human P2Y₂R (Applied Biosystems) using Lipofectamine 2000 (Invitrogen). A no-target siRNA was used as negative control. Forty-eight hours later, cells were treated with ATP with or without dexamethasone preincubation for 36 h. P2Y₂R mRNA expression was detected by RT-PCR to demonstrate successful silencing of the mRNA.

Nuclear Run-on Assay. HMEC-1 cells were treated with 100 nM dexamethasone and vehicle for 6 h. Nuclei were isolated using the Nuclei Pure Prep Nuclei Isolation Kit (Sigma-Aldrich). Transcription buffer containing 200 mM KCl, 20 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 4 mM dithiothreitol, 800 U of RNase inhibitor (Promega, Madison, WI), 20% glycerol, 4 mM ATP, 4 mM CTP, 4 mM GTP, 2 mM UTP, and 1.6 mM Biotin-16-UTP (Roche Applied Science, Indianapolis, IN) was added to the isolated nuclei solution. Transcription reactions were carried out at room temperature for 40 min and stopped by adding Buffer RL1, and then RNA was extracted as described above. Newly synthesized RNA transcripts were purified by using the μ MACS Streptavidin Kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. RNA expression levels were measured by real time RT-PCR.

Transient Transfection and Luciferase Assay. The human P2Y₂R promoter (prP2Y₂R-luc, -1572 to +93 base pairs) was kindly provided by F.-P. Gendron, Université de Sherbrooke, Sherbrooke, QC, Canada (Degagné et al., 2009). EA.hy926 cells at 80% confluence were seeded in 24-well plates for 24 h. Cells were transfected using FuGENE 6 Transfection reagent (Roche) with 0.1 μ g of P2Y₂R promoter construct or 0.1 μ g of pGL4.10 vector (control). pRL-TK (0.05 μ g) was cotransfected as internal control. Luciferase activity was measured using a Victor 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) after incubation with vehicle or 100 nM dexamethasone. Data were normalized to *Renilla reniformis* luciferase expression.

Cell Viability and Drug Cytotoxicity. A MultiTox-Fluor Multiplex Cytotoxicity assay (Promega) was performed to determine the cell viability and drug cytotoxicity after 8-h incubation with ATP (100 μ M), suramin (300 μ M), U73122 (1 μ M), Gö6976 (1 μ M), PD 98059 (20 μ M), SB 203580 (25 μ M), or SP 600125 (30 μ M) and after 36 h with dexamethasone alone and with ATP. None of these reagents affected cell viability (data not shown).

Statistical Analysis. Statistical analysis was performed on the mean values of independently conducted experiments. All experiments performed in vitro were repeated at least three times unless otherwise indicated. Data are presented as mean $\pm$ S.E.M. Significant differences between conditions were determined by a two-tailed Student's *t* test and one-way analysis of variance. A *p* value of <0.05 was considered significant.

Results

The Effects of Dexamethasone on LPS- and ATP-Induced IL-6 Release in HMEC-1 Cells. HMEC-1 cells were incubated in the presence or absence of 100 nM dexamethasone for 36 h and were then stimulated with vehicle,

LPS (10 ng/ml), ATP (100 μ M), or UTP (100 μ M) in fresh medium for 6 h. LPS-stimulated IL-6 release was suppressed by dexamethasone. In contrast, ATP- and UTP-induced IL-6 production was enhanced. Dexamethasone alone had no effect on IL-6 production (Fig. 1).

Dexamethasone Increases ATP-Induced IL-6 Production in a Time- and Dose-Dependent Manner in HMEC-1 Cells. Subconfluent HMEC-1 cells were incubated for 1, 2, 6, 12, or 24 h in depleted medium in the presence or absence of 100 μ M ATP. ATP induced the accumulation of IL-6 in a time-dependent manner (Fig. 2A). Likewise, the effect of dexamethasone and ATP on IL-6 production was also time-dependent, and the effects of dexamethasone preincubation occurred at 36 h (Fig. 2B). In subsequent studies, ATP incubation at 4 h and dexamethasone incubation at 36 h were selected to assess the mechanism associated with this response. The enhancement of ATP-induced IL-6 production by dexamethasone was dose-dependent with a minimum concentration of 10 nM and higher, reaching its maximum concentration at approximately 100 nM and then declining at 10,000 nM (Fig. 2C). Likewise, dexamethasone enhancement of IL-6 production induced by increasing concentrations of ATP occurred in a dose-dependent manner at concentrations of 1 μ M to 100 μ M and then declined at 1000 μ M (Fig. 2D).

Dexamethasone Up-Regulates ATP-Induced IL-6 mRNA Expression in HMEC-1 Cells. To investigate the effect of dexamethasone on ATP-induced IL-6 mRNA expression, quantitative real-time RT-PCR was performed. ATP induced an approximately 20-fold increase in the expression of IL-6 mRNA at 60 min (Fig. 3A). Preincubation with dexamethasone for 36 h up-regulated ATP-induced IL-6 mRNA almost 2-fold 4 h after ATP treatment (Fig. 3B).

The Effects of Dexamethasone on ATP-Induced IL-6 Depends on the Glucocorticoid Receptor. HMEC-1 cells were cultured with 10 μ M mifepristone (RU486), a glucocorticoid receptor antagonist, for 1 h followed by incubation with 100 nM dexamethasone for 36 h. After washing and incubation in fresh medium without hydrocortisone, IL-6 production in response to 100 μ M ATP was enhanced by dexamethasone (Fig. 4A). RU486 suppressed these dexamethasone

Fig. 1. Effect of dexamethasone on ATP-induced IL-6 release by HMEC-1 cells. HMEC-1 cells were cultured with or without the presence of dexamethasone (DEX; 100 nM) for 36 h, followed by the addition of vehicle, LPS (10 ng/ml), ATP (100 μ M), or UTP (100 μ M). LPS-induced IL-6 release was suppressed by dexamethasone (**, *p* < 0.01 versus without dexamethasone). In contrast, dexamethasone enhanced ATP- and UTP-induced IL-6 release (**, *p* < 0.01 versus without dexamethasone).

Fig. 2. Time and dose effects of ATP and dexamethasone on ATP-induced IL-6 release by HMEC-1 cells. **A**, HMEC-1 cells were incubated in depleted medium with ATP and vehicle for 1, 2, 6, 12, or 24 h. ATP increased IL-6 release after 24 h. **B**, dexamethasone increased ATP-induced IL-6 production in a time-dependent manner. HMEC-1 cells were incubated with 100 nM dexamethasone for 12, 24, or 36 h, followed by the addition of 100 μ M ATP for 6 h (**, $p < 0.01$ versus ATP alone; #, $p < 0.05$ versus DEX + ATP at 24 h). **C**, HMEC-1 cells were incubated with dexamethasone at concentrations of 1, 10, 100, 1000, and 10,000 nM for 36 h, followed by the addition of 100 μ M ATP for 6 h. Dexamethasone increases in ATP-induced IL-6 were dose-dependent, with a plateau effect at concentrations between 100 and 1000 nM ($p < 0.01$ versus ATP alone) and then a decline at 10,000 nM. **D**, increasing concentrations of ATP (1–1000 μ M) were associated with enhanced IL-6 production, and response was greatest at 100 μ M and then declined at 1000 μ M. Cells were incubated with 100 nM dexamethasone for 36 h, followed by the addition of ATP at concentrations of 1, 10, 100, and 1000 μ M for 6 h (**, $p < 0.01$ versus ATP alone).

Fig. 3. Effect of ATP on IL-6 mRNA induction by HMEC-1 cells. **A**, HMEC-1 cells were cultured with or without 100 μ M ATP for 5, 15, 30, 60, 120, 240, and 360 min, and mRNA expressions were measured by real-time RT-PCR. The data are shown as fold change of a parallel control. The maximum effect occurred at 60 min and returned to baseline by 240 min. **B**, HMEC-1 cells were cultured with 100 nM dexamethasone for 36 h, followed by 100 μ M ATP for 60, 120, 240, and 360 min. The fold change in IL-6 mRNA expression measured by real-time RT-PCR is shown compared with ATP alone. Dexamethasone maximum effects on IL-6 mRNA occurred 240 min after ATP stimulation.

Fig. 4. Dexamethasone enhancement of ATP-induced IL-6 is glucocorticoid receptor-dependent and requires P2Y₂R and downstream activation of PLC and p38 MAPK. **A**, HMEC-1 cells were cultured in vehicle and mifepristone (RU486; 10 μ M) 30 min before the addition of 100 nM dexamethasone for 36 h, followed by the addition of 100 μ M ATP. Supernatants were collected 6 h later and analyzed for IL-6 content. DEX enhancement of ATP-induced IL-6 production was inhibited by RU486 (**, $p < 0.01$ versus ATP alone; ##, $p < 0.01$ versus DEX with ATP). **B**, HMEC-1 cells were cultured in vehicle and the nonselective P2 receptor inhibitor suramin (300 μ M) for 30 min, followed by the addition of 100 μ M ATP for 6 h. Suramin significantly diminished the ATP induction of IL-6 production (**, $p < 0.01$ versus control; ##, $p < 0.01$ versus suramin with ATP). **C**, HMEC-1 cells were transfected with a negative control and P2Y₂ siRNA, and the P2Y₂ mRNA expression was measured by real-time RT-PCR. After 48 h of transfection, the cells were incubated with or without 100 nM dexamethasone for 36 h, followed by the addition of 100 μ M ATP for 6 h. Silencing of the P2Y₂ mRNA attenuated the ATP induction of IL-6 and the dexamethasone enhancement of this response (***, $p < 0.01$ versus the respective control). **D**, HMEC-1 cells were incubated with a phospholipase C inhibitor, U73122 (1 μ M); a phosphokinase C inhibitor, G66976 (1 μ M); an ERK1/2-related inhibitor, PD 98059 (20 μ M); a p38 MAPK inhibitor, SB 203580 (25 μ M); or a JNK inhibitor, SP 600125 (30 μ M), for 30 min, followed by the addition of 100 μ M ATP. Six hours later, IL-6 in supernatant was measured. Inhibition of the PLC and p38 MAPK pathways significantly decreased IL-6 release after ATP stimulation (***, $p < 0.001$ versus ATP alone). Inhibition of the ERK1/2 and JNK pathways was less effective in attenuating the ATP-induced IL-6 response, whereas PKC inhibition had no significant effect (**, $p < 0.01$ versus ATP alone).

effects, suggesting that the effect of dexamethasone is glucocorticoid receptor-dependent.

ATP-Induced IL-6 Production Is Mediated by the P2Y₂R Receptor in HMEC-1. HMEC-1 cells strongly express mRNA for the purinergic receptors P2X₄, P2X₅, P2X₇, P2Y₂R, and P2Y₁₁ and weakly express mRNA for P2X₁ and P2X₃ (Seiffert et al., 2006). ATP can bind to different P2 receptors with variable affinities. To characterize which receptor is involved in ATP-induced IL-6 production, we measured intracellular calcium mobilization induced by various nucleotides. ATP and UTP increased intracellular Ca²⁺ mobilization in HMEC-1 cells corresponding to EC₅₀ values of 364 $\pm$ 65 and 170 $\pm$ 32 nM, respectively. The EC₅₀ values for ADP and UDP were 16,800 $\pm$ 2590 and 3780 $\pm$ 1520 nM, respectively. Previous reports suggest that this agonist pro-

file is consistent with P2Y₂R activation (Nicholas et al., 1996). Suramin, a P2 receptor antagonist, significantly attenuated the ATP-induced IL-6 response (Fig. 4B). To characterize the specific P2 receptor associated with this response, we used siRNA inhibition with P2Y₂ siRNA that significantly attenuated ATP-induced IL-6 production (Fig. 4C).

ATP-Induced IL-6 Synthesis Is Triggered through PLC-Linked P2Y₂R Receptor Activation and Downstream Activation of p38 MAPK. The signaling mechanism of ATP-induced IL-6 synthesis in endothelial cells has not been well characterized. P2Y₂R is a G_q protein-coupled receptor with known downstream signaling through PLC, calcium influx, and PKC. We used specific inhibitors of PLC and PKC to assess their contribution to endothelial cell activation by ATP. ATP-induced IL-6 production was diminished

by 63.2% by incubation with the PLC inhibitor U73122 (1 μ M) but unaffected by the PKC inhibitor Gö6976 (1 μ M) (Fig. 4D). These data suggest a PLC-dependent process is involved in the ATP-induced increase of IL-6 in HMEC-1 cells. To elucidate whether MAPKs contribute to this response, three MAPK inhibitors were evaluated: an ERK1/2 inhibitor (PD 98059), a p38 MAPK inhibitor (SB 203580), and a JNK inhibitor (SP 600125). ATP-induced IL-6 production was abolished by SB 203580 ($88.1 \pm 0.48\%$, $p < 0.001$) (Fig. 4D). In contrast, PD 98059 and SP 600125 each caused a minor decrease ($14.8 \pm 1\%$ and $15.9 \pm 3.3\%$, respectively). These data suggest that ATP-induced IL-6 is triggered through a PLC-linked P2Y₂R activation with downstream receptor-mediated signals including p38 MAPK in HMEC-1 cells. An inhibitor of phosphatidylinositol 3-kinase, LY294002, was also applied but did not show any effect on ATP-induced IL-6 production (data not shown).

Dexamethasone Increases ATP-Induced Intracellular Ca²⁺ Release. To investigate the effect of dexamethasone on intracellular calcium ($[Ca^{2+}]_i$), ATP-induced $[Ca^{2+}]_i$ was measured after dexamethasone incubation. The effect of ATP on $[Ca^{2+}]_i$ was concentration-dependent between 1 nM and 100 μ M and was enhanced significantly by dexamethasone at 100 nM and 1 μ M, suggesting that the enhancement by dexamethasone of ATP-induced IL-6 production may arise in part from the $[Ca^{2+}]_i$ increase (Fig. 5).

Dexamethasone Up-Regulates the P2Y₂R Receptor mRNA Expression. Regarding the mechanisms of dexamethasone-induced increase of ATP-induced IL-6 production, we hypothesized that dexamethasone influences one or several steps involved in the PLC-linked P2Y₂R and p38 MAPK signal transduction pathway. HMEC-1 cells were incubated with dexamethasone or a parallel control for 2, 4, 6, 24, 36, and 48 h. P2Y₂R mRNA was up-regulated approximately 2-fold when incubated with dexamethasone for 6 h (Fig. 6A). P2Y₂R protein was increased after dexamethasone incubation for 36 h (Fig. 6B). The nuclear run-on assay revealed that dexamethasone enhanced the transcription rate of P2Y₂R mRNA synthesis approximately 1.5-fold by 40 min (Fig. 6C). Thus, dexamethasone is associated with an increased rate of transcription and accumulation of P2Y₂R mRNA, which contribute to enhanced IL-6 production in-

duced by ATP. Forty-eight hours after the human P2Y₂R promoter (prP2Y₂R-luc) was transfected into the EA.hy926 endothelial cell line, stimulation with dexamethasone for 6 h was associated with a dose-related increase in the luciferase reporter expression (Fig. 6D).

Enhancement of Additional ATP-Induced Mediators by Dexamethasone. To assess whether additional ATP-induced endothelial cell inflammatory responses were enhanced by dexamethasone, gene profiles of 84 genes associated with endothelial cell function were performed on cells treated for 36 h with dexamethasone and 1 and 4 h with ATP. Dexamethasone enhanced ATP-induced gene expression of six genes: *IL-6*, *TNF α* , *TNFAIP3*, *VCAM-1*, *ICAM-1*, and *SELE* (Fig. 7A). The interaction of dexamethasone and ATP had notable effects on mRNA induction in endothelial cells. Within 1 h of ATP stimulation, *IL-6*, *TNF α* , and *TNFAIP3* mRNA rose more than 2-fold compared with control. The addition of dexamethasone enhanced these increases in mRNA for these three genes and further enhanced the expression of *VCAM-1*. Differential expression of these genes at 4 h (Fig. 7B) after stimulation with ATP and dexamethasone showed a return toward baseline values of the mRNA for *IL-6* and *TNF α* and an induction of three adhesion molecules (*VCAM-1*, *ICAM-1*, and *SELE*) by ATP with an enhancement of their induction by dexamethasone to 8-fold or greater compared with baseline.

We performed a multiplex cytokine protein assay on endothelial supernatants after dexamethasone incubation for 36 h, followed by incubation with ATP for 6 h. Dexamethasone enhanced ATP-induced IL-8 production (Fig. 8A) but did not enhance the ATP-induced MCP-1 release (Fig. 8B). No changes were found in granulocyte colony-stimulating factor, IL-1 β , IL-1ra, IL-10, *TNF α* , or macrophage inflammatory protein-1 α protein secretion under these conditions (data not shown).

Discussion

We have observed that dexamethasone, a potent glucocorticoid, leads to an unanticipated enhancement of endothelial inflammatory mediator production by extracellular ATP. These effects are mediated in part by the direct effect on the P2Y₂R promoter, enhancing transcription of P2Y₂R mRNA and protein expression. Downstream signaling occurs predominantly through PLC and p38 MAPK pathways. Furthermore, the inflammation-promoting effects include enhanced release of IL-8, a key chemoattractant, and increased transcription of *ICAM-1*, *VCAM-1*, and *SELE*, adhesion molecules that play an important role in modulating inflammation and cell trafficking at the endothelial interface.

Despite the essential role of endogenous glucocorticoids in the stress response and their therapeutic use in acute inflammatory states such as septic shock, only limited data are available that define the effects of glucocorticoids on purinergic receptor responses initiated by ATP. Dexamethasone causes a rapid (e.g., 0.5–6 h) but transient up-regulation of P2Y₂R mRNA in murine thymocytes (Koshiba et al., 1997). Hydrocortisone inhibited adenosine 5'-(γ -thiotriphosphate) induction of CXCL8 (IL-8) in HMEC-1 endothelial cells when coincubated for 24 h, suggesting that the interval between glucocorticoid and ATP exposure may be important in subsequent responses (Bender et al., 2008). Nongenomic effects of

Fig. 5. Effect of dexamethasone on $[Ca^{2+}]_i$ in response to ATP. HMEC-1 cells were cultured in the presence of DEX at concentrations of 10, 100, or 1000 nM for 36 h, followed by the addition of 100 μ M ATP, and then $[Ca^{2+}]_i$ was measured immediately. The effect of ATP on $[Ca^{2+}]_i$ was concentration-dependent and enhanced by dexamethasone.

Fig. 6. Dexamethasone up-regulates the P2Y₂R expression in HMEC-1 cells. **A**, cells cultured with 100 nM dexamethasone for 2, 4, 6, 12, 24, 36, and 48 h increased P2Y₂R mRNA, with a maximal response by 6 h that remained elevated for 48 h. **B**, cells were incubated with dexamethasone for 48 h. P2Y₂R and β -actin were measured by Western blot. Dexamethasone increased P2Y₂R protein expression compared with control. **C**, the dexamethasone effect on P2Y₂R transcription was measured with nuclear run-on assay. Dexamethasone significantly induced P2Y₂R mRNA transcription after 6 h (*, $p < 0.05$). **D**, cells were transfected with P2Y₂R luciferase promoter for 48 h, followed by incubation with dexamethasone (10–1000 nM) for 6 h. Dexamethasone was associated with an increasing dose response in relative luciferase activity of the P2Y₂R-transfected promoter (*, $p < 0.05$).

glucocorticoids on purinergic signaling have been described in a variety of cell types. These responses occur within seconds or minutes and may inhibit or enhance downstream signaling. Preincubation with dexamethasone blocks ATP-stimulated calcium influx in murine neuroblastoma cells via a P2X mechanism (Han et al., 2005). In contrast, preincubation with dexamethasone results in a rapid enhancement of ATP-induced calcium influx and enhanced nitric oxide production in guinea pig spiral ganglion neurons via P2X activation. Within seconds of application, corticosterone inhibits rapid ATP-induced currents in dorsal root ganglions mediated via the P2X₃ receptor (Liu et al., 2008). Rat astrocytes stimulated with ATP analogs rapidly release arachidonic acid and subsequently differentiate with cell process elongation after three days (Bolego et al., 1997). These calcium-independent responses are blocked by suramin or dexamethasone, suggesting a P2Y receptor-linked response. Porcine endothelial cells have a rapid sustained rise in intracellular calcium in response to cortisol alone (Schneider et al., 1997). In contrast, incubation of human bronchial epithelial cells with dexamethasone inhibits ATP-induced calcium influx

and chloride efflux, suggesting a nongenomic antisecretory effect of dexamethasone (Urbach et al., 2002).

Several lines of evidence show that the density of purinergic receptors changes during the course of inflammatory responses (Bours et al., 2006). Best characterized are P2X₇ receptors on monocytes that are down-regulated by anti-inflammatory cytokines (e.g., IL-4, IL-10) and induced by TNF α or endotoxin. With the up-regulation of the P2X₇ receptors, inflammatory activation by endotoxin and interferon- γ induces a reciprocal down-regulation of the P2Y₂ receptor (Humphreys and Dubyak, 1996; Martin et al., 1997). This dynamic modulation of purinergic receptors has been described as the fine-tuning inflammatory and immune responses (Bours et al., 2006). Our data suggest that glucocorticoids are an additional factor that will modulate the inflammatory responses of endothelial cells to extracellular ATP. However, rather than suppressing inflammation, the presence of dexamethasone enhances inflammation initiated by ATP by increasing the expression level of the P2Y₂R and activation of its associated downstream PLC and p38 MAPK signaling pathways.

Fig. 7. Dexamethasone enhancement of ATP-induced gene expression. Cells were incubated with or without 100 nM dexamethasone for 36 h, followed by the addition of 100 μ M ATP for either 1 h (A) or 4 h (B). The mRNA expression was measured by real-time RT-PCR array. A threshold value of a 2-fold increase compared with control or ATP alone was considered significant with a nominal p value of <0.05 based on at least three independent experiments. The mean $\pm$ S.E.M. values for ATP compared with control (ATP/Control), dexamethasone compared with control (DEX/Control), dexamethasone and ATP compared with control (DEX ATP/Control), and dexamethasone and ATP compared with ATP alone (DEX ATP/ATP) are shown. Within 1 h of ATP stimulation, IL-6, TNF α , and TNFAIP3 mRNA rose more than 2-fold compared with control. A, dexamethasone enhanced these increases in mRNA for these three genes and enhanced the expression of VCAM-1. B, differential expression at 4 h after stimulation with ATP and dexamethasone showed a return of the mRNA toward baseline values for IL-6 and TNF α and induction of three adhesion molecules (VCAM-1, ICAM-1, and SELE) by ATP with enhancement of their induction by dexamethasone to 8-fold or greater compared with baseline.

Fig. 8. Dexamethasone enhances ATP-induced IL-8 secretion but not MCP-1. Cells were incubated with 100 nM dexamethasone for 36 h, followed by the addition of 100 μ M ATP for 6 h. A, ATP-induced IL-8 secretion, and this response was enhanced by dexamethasone (*, $p < 0.05$). B, MCP-1 release was induced by ATP but was not enhanced by dexamethasone.

Glucocorticoids may suppress or enhance cell inflammatory responses depending in part on the timing and context of target cell exposure. Gene profiles of human peripheral blood mononuclear cells after incubation with dexamethasone show that some genes associated with innate immunity are induced (e.g., receptors for chemokines, cytokines, Toll-like and scavenger receptors), whereas others are repressed (e.g., IL-1 β , IL-8, interferon- γ), suggesting that glucocorticoid modulation of genes associated with inflammation is not uniformly immunosuppressive (Galon et al., 2002). P2Y₂R responses associated with low increases in ATP concentra-

tions are mediated by this receptor because of its high affinity for ATP (e.g., $EC_{50} \leq 1 \mu$ M) (Bours et al., 2006; Trautmann, 2009), with effects on immune cells that include the induction of chemotaxis and reduced secretion of proinflammatory cytokines by dendritic cells (Bours et al., 2006; Trautmann, 2009). In contrast, responses to high levels of extracellular ATP (e.g., $EC_{50} > 100 \mu$ M) could potentially constitute a danger signal heralding cell injury. These effects are typically mediated by the P2X₇ receptor and include enhanced release of inflammatory cytokines, costimulation for antigenic stimulation of T and B lymphocytes, as well as

induction of apoptosis of cells (Bours et al., 2006; Trautmann, 2009). We show that the enhanced inflammatory response is present with ATP concentrations ranging from 1 to 1000 μ M (Fig. 2D). Our data suggest that exogenous glucocorticoids may amplify cell signaling initiated by a range of extracellular ATP concentrations binding to the P2Y₂R receptor, which augment rather than suppress the mRNA for inflammatory molecules and protein production.

The P2Y₂R has been implicated in key cellular targets that play essential roles in vascular inflammation, atherosclerosis, wound healing, and chronic inflammation (Bours et al., 2006; Erlinge and Burnstock, 2008). The contribution of the P2Y₂R to pathophysiologic responses is suggested by models of knockout and overexpression of the receptor. In a model of acute lung injury after cecal ligation and puncture, homozygous P2Y₂R knockout mice have impaired leukocyte migration into the peritoneal cavity, increased blood leukocyte counts, less lung tissue injury by histologic criteria, and reduced mortality (Inoue et al., 2008). Others have shown that double knockout mice P2Y₁R/P2Y₂R(−/−) have worse mortality, blunted cytokine responses, and increased susceptibility to *Pseudomonas aeruginosa* infection than either single P2Y₁R or P2Y₂R knockouts (Geary et al., 2005). Transgenic overexpression of the P2Y₂R using a lentivirus vector revealed lymphocytic infiltration of the lacrimal glands, a lymphocytic-associated glomerulonephropathy, and portal triad hepatitis (Agca et al., 2009).

IL-6 is a pleiotropic cytokine that is a key mediator of the acute phase response. It is secreted at sites of inflammation and initiates a transcriptional inflammatory response. IL-6, IL-1, and TNF α can stimulate adrenocorticotropin synthesis and activate the hypothalamic-pituitary-adrenal axis to secrete cortisol, which suppresses the release of these proinflammatory cytokines and thus illustrates an important negative feedback loop between the immune and neuroendocrine systems (Chesnokova and Melmed, 2002). Our findings suggest that in contrast glucocorticoids contribute to a positive feedback loop that amplifies inflammatory responses initiated by ATP, which may result in enhanced migration and activation of inflammatory cells to injured or infected tissue. This proinflammatory mechanism may be dampened by glucocorticoid induction of ectonucleotide pyrophosphatase/phosphodiesterase NPP1, resulting in extracellular nucleotide hydrolysis and recycling (Rebbe and Hickman, 1991; Goding et al., 2003). In addition, ATP stimulated the induction of TNFAIP3 mRNA (Fig. 7), a zinc finger protein that inhibits nuclear factor- κ B activation as well as TNF α -mediated apoptosis. Dexamethasone enhanced TNFAIP3 mRNA induction, suggesting an additional glucocorticoid-induced counterregulatory mechanism in endothelial inflammation (Heyninck and Beyaert, 2005).

Limitations of the current study include the in vitro focus of our experiments in specific endothelial cell lines. The generalizability of these responses to primary endothelial cells from different tissues remains to be characterized. Furthermore, hydrocortisone withdrawal on the endothelial cells 24 h before stimulation with dexamethasone and ATP may result in cell stress and represent an additional variable that may alter the cell phenotype for enhanced inflammatory mediator responses.

We have shown that dexamethasone leads to an unexpected enhancement of the production of endothelial cell inflammatory

mediators induced by extracellular ATP via a P2Y₂R-dependent mechanism. This positive feedback loop of glucocorticoids and ATP-induced endothelial inflammation highlights the observation that glucocorticoids are not uniformly immunosuppressive. Their effects on inflammatory responses in part depend on dose and timing of cell exposure (Galon et al., 2002). Our observations have implications for investigations that evaluate the role of purinergic receptors and ATP in the pathogenesis of vascular inflammation including shock syndromes, angiogenesis, and atherosclerosis.

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