Dexamethasone Enhances ATP-induced Inflammatory Responses

In Endothelial Cells

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Text pages 30

Tables 0

Figures 8

References 45

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Abstract - 239
Introduction – 601
Discussion – 1267

Abbreviations:

**DEX**, dexamethasone; **DMSO**, dimethyl sulfoxide; **ERK**, extracellular-signal-regulated kinase; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **G-CSF**, granulocyte colony-stimulating factor; **Gö6976**, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; **ICAM-1**, intracellular adhesion molecule-1; **IL**, interleukin; **IL-1ra**, IL-1 receptor antagonist; **JNK**, c-Jun N-terminal kinases; **LPS**, lipopolysaccharide; **LY294002**, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; **MAPK**, mitogen associated protein kinase; **MCP-1**, monocyte chemotactic factor; **mifepristone RU486**, (11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one); **MIP-1α**, macrophage inflammatory protein; **P2**, purinergic receptor 2; **PCR**, polymerase chain reaction; **PD 98059**, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; **PKC**, protein kinase C; **PLC**, phospholipase C; **RT-PCR**, reverse transcription polymerase chain reaction; **SB 203580**, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; **SELE**, E- selectin; **SP 600125**, 1,9-Pyrazoloanthrone, Anthrapyrazolone; **TNFα**, tumor necrosis factor; **TNFAIP3**, TNFα-induced protein 3; **U73122**, 1-[6-[(17β)-3-Methoxyestra-1, 3, 5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione; **VCAM-1**, vascular cell adhesion molecule-1
Abstract

The purinergic nucleotide adenosine 5’-triphosphate (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 describes the effects of glucocorticoids on ATP-induced inflammation. In a human microvascular endothelial cell line (HMEC-1), extracellular ATP induced 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 (U73122) (63.2 ± 3 %, p < 0.001) and abolished by a p38 mitogen activated protein kinase inhibitor (SB203580) (88 ± 1 %, p <0.001). Cells treated with dexamethasone induced mRNA expression of the purinergic P2Y2 receptor (P2Y2R) 1.8 ± 0.1 fold and when stimulated with ATP, enhanced Ca^{2+} release and augmented IL-6 mRNA expression. Silencing of the P2Y2R by its siRNA decreased ATP-induced IL-6 production by 81 ± 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. Further, 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. neurons) and non-excitable (i.e. endothelium, epithelium, myeloid) tissues (Abbracchio et al., 2006; Chen et al., 2006). ATP release may occur constitutively (Schwiebert et al., 2002) or following 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, adenosine diphosphate, bacterial components, 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 following tissue damage and necrosis and 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 activate a family of purinergic receptors (P2) 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). Following 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). While 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 dyslipidemias) and
progression of atherosclerotic cardiovascular disease (Walker, 2007). Low doses of
corticosteroids have become an important adjunctive therapy in the treatment of septic shock
(Minneci et al., 2009). Thus, endogenous and administered glucocorticoids have the potential to
affect a myriad number of host inflammatory responses. In order 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.

METHODS

Media and cell lines

The human dermal microvascular endothelial cell line-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 (FBS, Invitrogen, USA), epidermal growth factor (10 ng/ml, BD Biosciences, USA), hydrocortisone (HC, 1 µg/ml, Sigma-Aldrich, USA) and 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, USA), L-Glutamine (2 mmol/L, Invitrogen, USA) in a humidified atmosphere at 37°C with 5% CO₂. Hydrocortisone was removed 24 h prior to 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 (HUVEC) with A549 lung carcinoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 2 mmol/L 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, USA. Dexamethasone, RU486, U73122, Gö6976, PD 98059, SB 203580 and SP
600125 were dissolved in DMSO (Sigma-Aldrich, USA). 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, E. coli O111:B4, Ultra Pure) was purchased from List Biological Laboratories Inc (Campbell, CA, USA).

**Cytokine measurements**

HMEC-1 cells were cultured in 24 well plates (10⁵ cells/1 ml/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 System, USA) according to the manufacturer’s instructions. A multiplex assay for simultaneous measurement of nine secreted mediators (G-CSF, IL-1β, IL-6, IL-8, IL-10, TNFα, IL-1ra, MCP-1, MIP-1α) was performed using the Fluorokine MAP Human Base Kit A (R&D Systems, USA).

**Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in HMEC-1 cells**

Cells were cultured (10⁵ cells/ml) in 100 μl of medium in 96-well plates for 24 h and then treated under different conditions and intracellular calcium measured (FLIPR Calcium Assay Kit, Molecular Devices, Sunnyvale, CA, USA). Cells were loaded with 50 μl dye with probenecid in each well and incubated for 45 min at room temperature. The compound plate was prepared with dilutions of various compounds in Hank's 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 following 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 RNeasy total RNA Mini Kit as manufacturer’s instructions (Qiagen, USA). Total RNA (2 μg) was reverse-transcribed with High Capacity Reverse Transcription kit. cDNA was amplified by PCR using TaqMan probe for IL-6 and P2Y₂R. GAPDH was used as an endogenous control. Quantitative real-time PCR was performed using the TaqMan Universal PCR Master Mix. The PCR condition was denaturing at 95°C for 15s 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 5 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 and applied to 384 well plates that are pre-dispensed with gene specific primer sets using the Biomek 2000 (Beckman Coulter Inc). A two-step cycling program employed (1 cycle 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and one minute at 60°C ). 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 are determined by the two tailed Student’s \( t \)-test with a nominal \( p < 0.05 \) considered as significant.

**RNA interference-mediated down regulation of P2Y\(_2\)R**

The HMEC-1 cells were grown in 24-well plates at 30-50\% confluence for overnight then transfected with 75 pmol siRNA for human P2Y\(_2\)R (Applied Biosystems) using lipofectamine 2000 (Invitrogen). A no-target siRNA was used as negative control. 48 h later, cells were treated with ATP with or without dexamethasone preincubation for 36 h. P2Y\(_2\)R mRNA expression was detected by RT-PCR to demonstrate successful silencing of the mRNA.

**Nuclear run-on assay**

HMEC-1 cells were treated with dexamethasone (100 nM) and vehicle for 6 h. Nuclei were isolated using Nuclei Pure Prep Nuclei Isolation Kit (Sigma). Transcription buffer containing 200 mmol/L KCl, 20 mmol/L Tris-HCl (pH 7.5), 6 mmol/L MgCl\(_2\), 4 mmol/L DDT, 800 U RNase inhibitor (Promega), 20\% glycerol, 4 mmol/L ATP, 4 mmol/L CTP, 4 mmol/L GTP, 2 mmol/L UTP, 1.6 mmol/L Biotin-16-UTP (Roche) was added to the isolated nuclei solution. Transcription reactions were carried out at room temperature for 40 min and stopped by adding RLT buffer and then RNA extracted as described above. New synthesized RNA transcripts were purified by using \( \mu \)MACS Streptavidin Kit (Miltenyi Biotech, CA) according to the manufacturer’s instructions. RNA expression levels were measured by real time RT-PCR.

**Transient transfection and luciferase assay**

The human P2Y\(_2\)R promoter (prP2Y\(_2\)R-luc, -1572 to +93 bp) was kindly provided by
Prof. F-P Gendron, Université de Sherbrooke, Sherbrooke, QC, Canada (Degagne 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 µg of P2Y2R promoter construct or 0.1 µg of pGL4.10 vector (control). 0.05 µg pRL-TK was cotransfected as internal control. Luciferase activity was measured using Victor 1420 Multilabel Counter (Perkin Elmer) after incubation with vehicle or dexamethasone (100 nmol/L). Data were normalized to Renilla luciferase expression.

Cell viability and drug cytotoxicity

MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) was performed to determine the cell viability and drug cytotoxicity after 8 h-incubation with ATP (100 µmol/L), suramin (300 µmol/L), U73122 (1 µmol/L), Gö6979 (1 µmol/L), PD 98058 (20 µmol/L), SB 203580 (25 µmol/L) or SP 600125 (30 µmol/L) and 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 3 times, unless otherwise indicated. Data are presented as mean ± sem. Significant differences between conditions were determined by the 2-tailed student t-test and 1-way ANOVA. A p < 0.05 was considered to be significant.
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 dexamethasone (100 nmol/L) for 36 h, and then stimulated with vehicle, LPS (10 ng/ml), ATP (100 µmol/L) or UTP (100 µmol/L) 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 (Figure 1).

Dexamethasone increases ATP induced IL-6 production in a time- and dose-dependent manner in HMEC-1 cells

Sub-confluent HMEC-1 cells were incubated for 1, 2, 6, 12 or 24 h in depleted medium in the presence or absence of 100 µmol/L ATP. ATP induced the accumulation of IL-6 in a time-dependent manner (Figure 2A). Similarly, the effect of dexamethasone and ATP on IL-6 production was also time-dependent and the effects of dexamethasone pre-incubation occurred at 36 h (Figure 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 nmol/L and higher, reaching maximum at approximately 100 nmol/L and then declined at 10,000 nmol/L (Figure 2C). Similarly, dexamethasone enhancement of IL-6 production induced by increasing concentrations of ATP occurred in a dose-dependent manner at concentrations of 1 µmol/L to 100 µmol/L and then declined at 1000 µmol/L (Figure 2D).

Dexamethasone upregulates 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 (Figure 3A). Preincubation with dexamethasone for 36 h upregulated ATP-induced IL-6 mRNA almost 2-fold, 4 h after ATP treatment (Figure 3B).

The effects of dexamethasone on ATP-induced IL-6 depends on the glucocorticoid receptor

HMEC-1 cells were cultured with mifepristone (RU486) (10 µmol/L) a glucocorticoid receptor antagonist for 1 h followed by incubation with 100 nmol/L dexamethasone for 36 h. After washing and incubation in fresh medium without hydrocortisone, IL-6 production in response to ATP 100 µmol/L was enhanced by dexamethasone (Figure 4A). RU486 suppressed these dexamethasone 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 receptors and weakly express mRNA for P2X1 and P2X3 receptors (Seiffert et al., 2006). ATP can bind to different P2 receptors with variable affinities. In order 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 Ca\(^{2+}\) mobilization in HMEC-1 cells corresponding to EC\(_{50}\) values of 364 ± 65 and 170 ± 32 nmol/L, respectively. The EC\(_{50}\) values for ADP and UDP were 16800 ± 2590 and 3780 ± 1520 nmol/L, respectively. Previous reports suggest that this agonist profile is consistent with P2Y2R activation (Nicholas et al., 1996). Suramin, a P2 receptor antagonist significantly
attenuated the ATP-induced IL-6 response (Fig 4B). In order to characterize the specific P2 receptor associated with this response, we used siRNA inhibition with P2Y₂R siRNA that significantly attenuated ATP-induced IL-6 production (Figure 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 µmol/L), but unaffected by the PKC inhibitor, Gö 6979 (1 µmol/L) (Figure 4D). These data suggest a PLC-dependent process is involved in the ATP-induced increase of IL-6 in HMEC-1 cells. To elucidate if 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 ± 0.48 %, p <0.001) (Figure 4D). In contrast, PD 98059 or SP 600125 caused a minor decrease (14.8 ± 1 % and 15.9 ± 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-induce IL-6 (data not shown).

**Dexamethasone increases ATP-induced intracellular Ca^{2+} release**
To investigate the effect of dexamethasone on \([\text{Ca}^{2+}]_i\), ATP-induced \([\text{Ca}^{2+}]_i\) was measured after dexamethasone incubation. The effect of ATP on \([\text{Ca}^{2+}]_i\) was concentration-dependent over the range between 1 nmol/L and 100 μmol/L and was enhanced significantly by dexamethasone at 100 nmol/L and 1 μmol/L, suggesting that the enhancement by dexamethasone of ATP-induced IL-6 production may arise in part from the \([\text{Ca}^{2+}]_i\) increase (Figure 5).

**Dexamethasone upregulates the P2Y\(_2\)R 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 PLC-linked P2Y\(_2\)R 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. P2Y\(_2\)R mRNA was upregulated approximately 2-fold when incubated with dexamethasone for 6 h (Figure 6A). P2Y\(_2\)R protein was increased after dexamethasone incubation for 36 h (Figure 6B). The nuclear run-on assay revealed that dexamethasone enhanced the transcription rate of P2Y\(_2\)R mRNA synthesis approximately 1.5-fold by 40 min (Figure 6C). Thus, dexamethasone is associated with an increased rate of transcription and accumulation of P2Y\(_2\)R mRNA that contribute to enhanced IL-6 production induced by ATP. Forty-eight hours after the human P2Y\(_2\)R promoter (prP2Y\(_2\)R-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 (Figure 6D).

**Enhancement of additional ATP-induced mediators by dexamethasone**

In order 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 h and 4 h with ATP. Dexamethasone enhanced ATP-induced gene expression of six genes: IL-6, TNFα, TNFAIP3, VCAM-1, ICAM-1, and SELE (Figure 7A). The interaction of dexamethasone and ATP further had notable effects on mRNA induction in endothelial cells. Within one hour of ATP stimulation, IL-6, TNFα and TNFAIP3 mRNA rose more than 2-fold compared to 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 (Figure 7B) after the stimulation with ATP and dexamethasone showed a return toward baseline values of the mRNA for IL-6 and TNFα 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 to baseline.

We performed a multiplex cytokine protein assay on endothelial supernatants after dexamethasone incubation for 36 h and followed by ATP for 6 h. Dexamethasone enhanced ATP-induced IL-8 (Figure 8A) but did not enhance the ATP-induced MCP-1 release (Figure 8B). No changes were found in G-CSF, IL-1β, IL-1ra, IL-10, TNFα, or MIP-1α 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 predominately through PLC and p38 MAPK pathways. Further, 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 defining the effects of glucocorticoids on purinergic receptor responses initiated by ATP. Dexamethasone causes a rapid (e.g., 0.5 to 6 h) but transient upregulation of P2Y2R mRNA in murine thymocytes (Koshiba et al., 1997). Hydrocortisone inhibited ATPγS induction of CXCL8 (IL-8) in HMEC-1 endothelial cells when co-incubated for 24 h suggesting that the interval between glucocorticoid and ATP exposure may be important in subsequent responses (Bender et al., 2008). Non-genomic effects of 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 analogues rapidly release arachidonic acid and subsequently differentiate with cell process elongation after 3 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 non-genomic anti-secretory 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 downregulated by anti-inflammatory cytokines (e.g., IL-4, IL-10) and induced by TNFα or endotoxin. With the upregulation of the P2X7 receptors, inflammatory activation by endotoxin and interferon gamma induces a reciprocal downregulation 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.

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) while others are repressed (e.g., IL-1β, IL-8, IFNγ) 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 concentrations are mediated by this receptor with high affinity for ATP (e.g., median effective concentration \( EC_{50} \leq 1\mu\text{mol/L} \) P2Y2R) (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$_{50}$ > 100µmol/L) potentially constitute a danger signal heralding cell injury. These effects are typically mediated by the P2X$_7$ receptor and include enhanced release of inflammatory cytokines, co-stimulation 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 µM to 1000 µM (Figure 2D). Our data suggest that exogenous glucocorticoids may amplify cell signaling initiated by a range of extracellular ATP concentrations binding to the P2Y$_2$R receptor, which augment rather than suppress the mRNA for inflammatory molecules and protein production.

The P2Y$_2$R 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 P2Y$_2$R to pathophysiologic responses is suggested by models of knockout and overexpression of the receptor. In a model of acute lung injury following cecal ligation and puncture, homozygous P2Y$_2$R 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 P2Y$_1$R/ P2Y$_2$R -/- have worse mortality, blunted cytokine responses and increased susceptibility to Ps. aeruginosa infection than either single P2Y$_1$R or P2Y$_2$R knockouts (Geary et al., 2005). Transgenic overexpression of the P2Y$_2$R 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, TNFα can stimulate ACTH 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. It may result in enhanced migration and activation of inflammatory cells to injured or infected tissue. This pro-inflammatory mechanism may be dampened by glucocorticoid induction of ecto-nucleotide 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 (Figure 7), a zinc finger protein that inhibits NF-kappa B activation as well as TNFα-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. Further, hydrocortisone withdrawal on the endothelial cells 24 hrs prior to 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 are in part dependent 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.
References


Footnotes

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Reprint requests

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Figure Legends

Figure 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 nmol/L) for 36 h followed by addition of vehicle, LPS (10 ng/ml), ATP (100 µmol/L) or UTP (100 µmol/L). LPS-induced IL-6 release was suppressed by dexamethasone (**p<0.01 vs. without dexamethasone). In contrast, dexamethasone enhanced ATP- and UTP-induced IL-6 release (**p<0.01 vs. without dexamethasone).

Figure 2 (A-D). 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, 24 hours. ATP increased IL-6 release over 24 h.

B) Dexamethasone increased ATP-induced IL-6 production in a time-dependent manner. HMEC-1 cells were incubated with dexamethasone (100 nmol/L) for 12, 24 or 36 h followed by addition of ATP (100 µmol/L) for 6 h (**p<0.01 vs. ATP alone, #p<0.05 vs. DEX + ATP at 24 h).

C) HMEC-1 cells were incubated with dexamethasone at 1, 10, 100, 1000, 10000 nmol/L for 36 h followed by addition of ATP (100 µmol/L) for 6 h. Dexamethasone increases in ATP-induced IL-6 was dose-dependent with a plateau effect at 100 – 1000 nmol/L concentration (p<0.01 vs. ATP alone) and then declined at 10,000 nmol/L.

D) Increasing concentrations of ATP (1 - 1000 µmol/L) was associated with enhanced IL-6 production and response was greatest at 100 µmol/L and then declined at 1000 µmol/L.
Dexamethasone 100 nmol/L for 36 h followed by addition of ATP at 1, 10, 100, 1000 µmol/L for 6 h (**p<0.01 vs. ATP alone).

**Figure 3 (A and B). Effect of ATP on IL-6 mRNA induction by HMEC-1 cells**

A) HMEC-1 cells were cultured with or without ATP (100 µmol/L) for 5, 15, 30, 60, 120, 240, 360 min and mRNA expressions 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 dexamethasone (100 nmol/L) for 36 h followed by ATP 100 µmol/L for 60, 120, 240, 360 min. The fold change in IL-6 mRNA expression measured by real-time RT-PCR is shown compared to ATP alone. Dexamethasone maximum effects on IL-6 mRNA occurred 240 min after ATP stimulation.

**Figure 4 (A-D). 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 µmol/L) 30 min prior to dexamethasone (100 nmol/L) for 36 h and followed by addition of ATP 100 µmol/L. 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 vs. ATP alone, ##p<0.01 vs. DEX with ATP).
B) HMEC-1 cells were cultured in vehicle and the non-selective P2 receptor inhibitor suramin (300 µmol/L) 30 min followed by ATP (100 µmol/L) for 6 h. Suramin significantly diminished the ATP-induction of IL-6 production ( **p<0.01 vs. control, # p<0.01 vs. suramin with ATP).

C) HMEC-1 cells were transfected with a negative control and P2Y2R siRNA and the P2Y2R mRNA expression was measured by real-time RT-PCR. After 48 h of transfection, the cells were incubated with or without dexamethasone 100 nmol/L for 36 h and then followed by ATP (100 µmol/L) for 6 h. Silencing of the P2Y2R mRNA attenuated the ATP-induction of IL-6 and the dexamethasone enhancement of this response (***p<0.01 vs. the respective control).

D) HMEC-1 cells were incubated with a phospholipase C inhibitor, U73122 (1 µmol/L), a phosphokinase C inhibitor, Gö6979 (1 µmol/L), an ERK1/2 related inhibitor, PD 98058 (20 µmol/L), a p38 MAPK inhibitor, SB 203580 (25 µmol/L) or a JNK inhibitor, SP 600125 (30 µmol/L), for 30 min followed by ATP (100 µmol/L). 6 h 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 vs. 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 vs. ATP alone).

**Figure 5. Effect of dexamethasone on [Ca^{2+}]_i in response to ATP**

HMEC-1 cells were cultured in the presence of DEX at 10, 100, 1000 nmol/L for 36 h followed by addition of ATP 100 µmol/L and then [Ca^{2+}]_i was measured immediately. The effect of ATP on [Ca^{2+}]_i was concentration-dependent and was enhanced by dexamethasone.

**Figure 6 (A-D). Dexamethasone upregulates the P2Y2R expression in HMEC-1**
A) Cells cultured with dexamethasone (100 nmol/L) for 2, 4, 6, 12, 24, 36, 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 beta actin were measured by Western blot. Dexamethasone increased P2Y2R protein expression compared to control.

C) 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 and followed by incubation with dexamethasone (10 - 1000 nmol/L) for 6 h. Dexamethasone was associated with an increasing dose-response in relative luciferase activity of the P2Y2R-transfected promoter (* p < 0.05).

Figure 7. Dexamethasone enhancement of ATP-induced gene expression

Cells were incubated with or without dexamethasone (100 nmol/L) for 36 h and followed by ATP (100 µmol/L) 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 to control or ATP alone was considered significant with a nominal p value <0.05 based on at least 3 independent experiments. The mean ± sem value for ATP compared to control (ATP/Control), dexamethasone compared to control (DEX/Control), dexamethasone and ATP compared to control (DEX-ATP/Control), and dexamethasone and ATP compared to ATP alone (DEX-ATP/ATP) are shown. Within one hour of ATP stimulation, IL-6, TNFα and TNFAIP3 mRNA rose more than 2-fold compared to control. Dexamethasone enhanced these increases in mRNA for these three genes and enhanced the expression of VCAM-1 (Figure 7A). Differential expression at 4 h (Figure 7B) after the stimulation with ATP and dexamethasone showed a return
of the mRNA towards baseline values for IL-6 and TNFα 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 to baseline.

**Figure 8. Dexamethasone enhances ATP-induced IL-8 secretion but not MCP-1**

Cells were incubated with dexamethasone (100 nmol/L) for 36 h and followed by ATP (100 µmol/L) for 6 h. ATP-induced IL-8 (A) secretion, and this response was enhanced by dexamethasone (*p<0.05). MCP-1 release (B) was induced by ATP but was not enhanced by dexamethasone.
Figure 3

A. Relative IL-6 mRNA Expression

Fold Change / Control

(min)

B. Relative IL-6 mRNA Expression

Fold Change / ATP

(min)
Figure 5

![Graph showing the relationship between RFU and log [ATP], M. The graph includes data points and error bars for different concentrations of ATP: Control, DEX 10 nM, DEX 100 nM, and DEX 1000 nM.](image-url)
Figure 7

A.

IL-6
TNF
ICAM1
VCAM1
SELE
TNFAIP3

Fold Change

B.

IL-6
TNF
ICAM1
VCAM1
SELE
TNFAIP3

Fold Change

Legend:
- ATP/Control
- DEX/Control
- DEX-ATP/Control
- DEX-ATP/ATP
Figure 8

A. IL-8 in Supernatant (pg/ml)

B. MCP-1 in Supernatant (pg/ml)

- DEX - +

ATP 100 μmol/L