PRIMING OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION PROTEINS FOR CYTOKINE-TRIGGERED POLYUBIQUITYLATION AND DEGRADATION BY THE A₂A ADENOSINE RECEPTOR

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ABSTRACT

Here we demonstrate that overexpression of the human A\textsubscript{2A} adenosine receptor (A\textsubscript{2A}AR) in vascular endothelial cells confers an ability of interferon-\(\alpha\) and a soluble IL-6 receptor/IL-6 (sIL-6R\(\alpha\)/IL-6) trans-signaling complex to trigger the down-regulation of signal transducer and activator of transcription (STAT) proteins. Interestingly, STAT down-regulation could be reversed by co-incubation with A\textsubscript{2A}AR-selective inverse agonist ZM241385 but not adenosine deaminase, suggesting that constitutive activation of the receptor was responsible for the effect. Moreover, STAT down-regulation was selectively abolished by proteasome inhibitor N-(benzyloxy carbonyl)leucinylleucinylleucinal (MG132) while lysosome inhibitor chloroquine was without effect. Down-regulation required Janus kinase (JAK) activity and a Tyr705\(\rightarrow\)Phe-mutated STAT3 was resistant to the phenomenon, suggesting that JAK-mediated phosphorylation of this residue is required. Consistent with this hypothesis, treatment of A\textsubscript{2A}AR-overexpressing cells with sIL-6R\(\alpha\)/IL-6 triggered the accumulation of polyubiquitylated wild-type but not Tyr705\(\rightarrow\)Phe-mutated STAT3. Support for a functional role of this process was provided by the observation that A\textsubscript{2A}AR overexpression attenuated the JAK/STAT-dependent up-regulation of vascular endothelial growth factor receptor-2 by sIL-6R\(\alpha\)/IL-6. Consistent with a role for endogenous A\textsubscript{2A}ARs in regulating STAT protein levels, prolonged exposure of endogenous A\textsubscript{2A}ARs in endothelial cells with ZM241385 \textit{in vitro} triggered the up-regulation of STAT3, while deletion of the A\textsubscript{2A}AR \textit{in vivo} potentiates STAT1 expression and phosphorylation. Together, these experiments support a model whereby the A\textsubscript{2A}AR can prime JAK-phosphorylated STATs for polyubiquitylation and proteasomal degradation, and represents a new mechanism by which an anti-inflammatory seven transmembrane receptor can negatively regulate JAK/STAT signaling.
Vascular endothelial cells (ECs) comprise a non-thrombotic anti-coagulatory surface that resists the onset of inflammation. The shift to a predominantly pro-inflammatory or "dysfunctional" state in response to injury or infection is triggered by a variety of stimuli, including pathogen-derived molecules, bioactive lipids and cytokines (Gimbrone, 1995; von der Thusen et al., 2003). Endothelial dysfunction is also strongly linked to the development of obesity and type II diabetes and underlies the increased susceptibility to cardiovascular disease displayed by individuals with these conditions (Fantuzzi and Mazzone, 2007; Ritchie et al., 2004). The development of the pro-inflammatory phenotype is now thought to be driven largely by the concerted action of so-called “adipocytokines” released from adipose tissue. Many studies have found that levels of several of these adipocytokines are chronically elevated in obese and diabetic subjects (Ritchie et al., 2004; Tilg and Moschen, 2006; Fantuzzi and Mazzone, 2007) and that those which have largely pro-inflammatory/atherogenic effects, such as IL-6 and leptin, can accumulate within atherosclerotic plaques and at sites of vascular injury (Schieffer et al., 2000; Schafer et al., 2004).

IL-6 exerts its effects on target cells by binding to either cell membrane-localised or soluble receptor α-chains. The receptor-cytokine complex then activates a dimeric transmembrane signal transducing component termed “gp130”. This allows constitutively bound tyrosine kinases of the “Janus kinase” (JAK) family to transphosphorylate and activate each other prior to phosphorylating multiple tyrosine residues on gp130 to enable docking of specific members of the “signal transducer and activator of transcription” (STAT) family as well as the protein tyrosine phosphatase SHP-2 via their SH2 domains (Heinrich et al., 2003). Recruitment of STAT1 and STAT3 causes their phosphorylation on Tyr701 and Tyr705 respectively by gp130-associated JAKs, resulting in their homo/heterodimerisation and translocation to the nucleus where they can initiate cytokine-inducible target gene transcription (Levy and Darnell, 2002).
It is becoming increasingly apparent that pro-inflammatory signaling pathways are also subject to regulation by non-cytokine stimuli, thus providing a means by which otherwise distinct signaling modules can negatively control cytokine responsiveness. The seven transmembrane (7TM) A2A adenosine receptor (A2AAR) has emerged as an important suppressor of vascular inflammation \textit{in vivo} (McPherson et al., 2001; Sitkovsky et al., 2004), largely due to receptor expression in neutrophils, monocytes, macrophages and other inflammatory cell types. For example, A2AAR-selective agonists can inhibit activation of the neutrophil respiratory burst (Sullivan et al., 2001) and elastase release (Anderson et al., 2000) in response to chemotactic peptide N-formylmethionyl-leucylphenylalanine. A2AAR activation can also mediate some of the suppressive effects of adenosine on pro-inflammatory aspects of macrophage function, such as IL-12 production (Haskó et al., 2000), while also enhancing the CCAAT/enhancer binding protein (C/EBP)-dependent induction of anti-inflammatory cytokine IL-10 (Csóka et al., 2007). Functional A2AARs expressed in vascular ECs also have important anti-inflammatory roles, including inhibition of adhesion molecule expression and monocyte adhesion (Sands et al. 2004; Zernecke et al., 2006) One aspect of the A2AAR’s effects is an ability to inhibit pro-inflammatory NF-κB activation by multiple cell type-specific mechanisms (Majumdar and Aggarwal, 2003; Sands et al., 2004). However, given its potent anti-inflammatory effects \textit{in vivo}, it is likely that the receptor inhibits additional pro-inflammatory signalling mechanisms to limit inflammation and associated tissue damage.

In this study, we have examined the effect of A2AAR overexpression on activation of the JAK/STAT pathway. We demonstrate that the A2AAR suppresses STAT phosphorylation in response to multiple cytokines by priming JAK-phosphorylated STATs for ubiquitylation and proteasomal degradation. This reveals a previously unappreciated mechanism by which it may be possible to suppress pro-inflammatory signaling in the vascular endothelium.
MATERIALS AND METHODS

Materials  The generation of plaque-purified adenoviruses (AV) encoding myc epitope-tagged human A2AAR and GFP have been described previously by us (Sands et al., 2004). AVs encoding Flag epitope-tagged WT and Tyr705→Phe mutated murine STAT3 were generously donated by Brian Foxwell (Kennedy Institute of Rheumatology, U.K.) and Keiko Yamauchi-Takahara (Osaka University Health Care Centre, Japan) (Kunisada et al., 1998; Williams et al., 2004). A2AAR knockout (KO) mice and their wild-type (WT) littermates (both on a CD1 background) were generated in a pathogen-free facility using founder heterozygotes. Offspring were genotyped by tail-tipping and PCR amplification of genomic DNA (Ledent et al., 1997). Anti-Flag M5 antibody and M2 antibody-conjugated Sepharose beads were from Sigma-Aldrich. Anti-ubiquitin antibody (sc-9133) was from Santa Cruz Biotechnology. Sources of other materials have been described elsewhere (Sands et al., 2004; Sands et al., 2006).

Cell culture and AV infection  HUVECs were propagated at 37°C in a humidified atmosphere containing 5% (v/v) CO2 in ECM-2 medium supplemented with 2% (w/v) fetal bovine serum, hydrocortisone, ascorbate and recombinant growth factors as recommended by the supplier (Cambrex Biosciences, Nottingham, U.K.). Human embryonic kidney (HEK) 293 cells for AV propagation were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, L-glutamine, penicillin and streptomycin. For infection with recombinant AVs, HUVECs were washed in regular growth medium and then incubated overnight with the same medium supplemented with recombinant AV at the multiplicities of infection (MOI) indicated in the Results. The next day, the virus-containing medium was aspirated and replaced with normal medium. Cells were used for analysis twenty-four hours later.
Treatment of mice with endotoxin  Endotoxic shock in age-matched WT and A2AAR KO mice was induced by intravenous injection of 0.4 mg/kg 0111:B4 E. coli lipopolysaccharide (LPS). Mice injected with PBS vehicle were used as injection controls. After 4 hours, animals were sacrificed for multiplex analysis of serum cytokine levels and isolation of the aorta for preparation of samples for SDS-PAGE.

Immunoblotting  Aortae isolated from vehicle or LPS-treated WT and A2AAR KO mice following sacrifice were frozen in liquid nitrogen and pulverised using a pestle and mortar. Pulverised extracts were then lysed directly in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer prior to analysis. Confluent HUVECs in six-well plates were treated as described in the figures prior to washing in ice-cold PBS and solubilisation by scraping into 50 μl/well detergent lysis buffer (50 mM sodium HEPES, pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium phosphate, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1 mM phenylmethysulfonyl fluoride (PMSF), 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine and EDTA-free complete protease inhibitor mix). Following brief vortexing, insoluble material was removed by microcentrifugation and the supernatant assayed for protein content using a bicinchonnic acid assay. Samples equalised for protein content (typically 10-20 μg/sample) were fractionated by SDS-PAGE. Following transfer to nitrocellulose, membranes were blocked for one hour at room temperature in blocking solution (5% (w/v) skimmed milk in TBS containing 0.1% (v/v) Tween-20 (TBST)). Membranes were then incubated either overnight at 4°C or for one hour at room temperature with primary antibody diluted to a final concentration of 1 μg/ml in 5% (w/v) IgG-free bovine serum albumin (BSA) in TBST. Following three washes in blocking solution, membranes were incubated for one hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody at a 1 in 1000 dilution in BSA/TBST. After further washes with TBST and TBS,
immunoreactive proteins were visualised by enhanced chemiluminescence. Quantification was by densitometric scanning of non-saturating exposed films using TotalLab v2.0 imaging software (Phoretix).

**Immunoprecipitation** Confluent HUVECs in six well dishes were pre-incubated with 6 μM MG132 prior to treatment with or without sIL-6Ra/IL-6 as described in the figure legends prior to termination of the incubation by placing dishes on ice and washing cell monolayers three times with ice-cold PBS. Cells were solubilised by scraping into 0.1 ml denaturing lysis buffer (50 mM sodium HEPES, pH 7.5, 100 mM sodium chloride, 1 mM N-ethylmaleimide, 2% (w/v) SDS, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine and EDTA-free complete protease inhibitor mix) and heating to 95°C for 5 min followed by probe sonication. After the addition of 0.9 ml lysis buffer containing Triton X-100 and sodium deoxycholate to give final concentrations of 1% (v/v) and 0.5% (w/v) respectively, insoluble material was removed by centrifugation and soluble fractions equalised for protein content and volume prior to incubation for 1 hr at 4°C with rotation with 25 μl packed volume of protein A-Sepharose beads in the presence of 0.2% (w/v) IgG-free BSA. Anti-STAT3 antibody (2μg/sample) was then added and the incubation continued for a further 1 hr. Immune complexes were isolated by brief centrifugation and washed three times with detergent lysis buffer prior to elution of precipitated proteins by the addition of electrophoresis sample buffer. Samples were then fractionated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Recombinant Flag-tagged STAT3 was immunoprecipitated by the addition of 20 μl packed volume of anti-Flag M2-Sepharose beads and incubation with rotation for 1 hr at 4°C prior to analysis by SDS-PAGE and immunoblotting as described above.

**Statistical analysis** Data are presented in the text as means ± SD for the number of experiments indicated, while representative experiments are shown in the figures.
Concentration-response data were fitted to a sigmoid curve using Prism 4 software (GraphPad, San Diego, CA). Statistical significance was assessed by either paired Student t tests or ANOVA using Instat 3 software (GraphPad).
RESULTS

Effect of A2AAR overexpression and activation on cytokine activation of the JAK/STAT pathway. A recombinant adenovirus (AV) was used to drive overexpression of myc epitope-tagged human A2AARs in HUVECs. Consistent with our previous study (Sands et al., 2004), 9E10 anti-myc immunoblotting revealed that recombinant A2AARs migrated as a doublet of 42 and 49 kDa (Figure 1A). Analysis of receptor function in control GFP-expressing cells demonstrated that treatment with the A2AAR-selective agonist CGS21680 (Jarvis et al., 1989) produced a transient increase in ERK1,2 phosphorylation, consistent with the presence of endogenous functional A2AARs (Sexl et al., 1997; Sands et al., 2004). However overexpression of recombinant A2AARs potentiated ERK1,2 phosphorylation at each time point (Figure 1A), demonstrating that the recombinant receptor was functional with respect to its ability to couple to downstream signalling pathways in HUVECs.

We have previously described how potentiating A2AAR overexpression over endogenous levels is sufficient to suppress NF-κB activation even in the absence of agonist (Sands et al., 2004). Thus we initially tested the effect of A2AAR overexpression on cytokine activation of the JAK/STAT pathway. In control cells, treatment with a sIL-6Rα/IL-6 trans-signaling complex produced a transient increase in the phosphorylation of STAT3 on Tyr705 which plateaued at 15 and 30 min. In comparison, overexpression of the A2AAR significantly reduced STAT phosphorylation at each time point (Figure 1B). Interestingly, while total levels of STAT3 were unaltered by sIL-6Rα/IL-6 treatment of GFP-expressing cells, a marked decrease in the amount of total STAT3 in A2AAR-expressing HUVECs that reached statistical significance 30 min post-cytokine stimulation (Figure 1B). A similar inhibitory effect of the A2AAR on total STAT1 expression and its cytokine-stimulated phosphorylation on Tyr701 was also observed (data not shown). Importantly, cell viability assays revealed
that the decrease in STAT levels did not simply reflect reduced HUVEC viability following A2AAR overexpression and cytokine treatment (data not shown).

The effect of on STAT down-regulation was then characterised in more detail. First, we examined the pharmacology of the response using the A2AAR-selective agonist CGS21680 and selective inverse agonist ZM241385 (Poucher et al., 1995; Klinger et al., 2002) on STAT protein levels. Co-incubation of sIL-6Rα/IL-6 with either drug did not produce any significant changes in STAT3 expression in control GFP-expressing cells. In contrast, sIL-6Rα/IL-6 treatment of A2AAR-overexpressing cells promoted reductions in both Tyr705-phosphorylated and total STAT3 which were significantly reversed by co-incubation with inverse agonist ZM241385. Co-incubation with the agonist CGS21680 did not further potentiate the effect of A2AAR overexpression, suggesting that the receptor displays a level of constitutive activity that is sufficient to observe maximal cytokine-stimulated down-regulation of STATs (Figure 2A). Again, the effect was not restricted to STAT3 as an identical pattern was also observed for STAT1 (data not shown). Additional experiments revealed that ZM241385-mediated reversal of the effect occurred in a concentration-dependent manner (Figure 2B; EC50 = 4.9±3.0 nM) consistent with the reported low nM affinity of the compound at the human A2AAR (Gao et al., 2000). In contrast, pre-treatment with 3U/ml adenosine deaminase (ADA) failed to alter the effect of A2AAR overexpression (58±15% decrease in total STAT3 in the absence of ADA versus 47±16% in the presence of ADA, p>0.05, n=3; Figure 2C), confirming that the ability of receptor overexpression to prime STATs for down-regulation was due largely to a degree of constitutive activity of the receptor rather than accumulation of the physiological agonist adenosine during the incubation.

To determine whether this effect was restricted to sIL-6Rα/IL-6 we also tested the effect of A2AAR overexpression on responses to interferon α (IFNα), which also activates STAT1 and
STAT3 (Brierley and Fish, 2002). Similar to sIL-6Rα/IL-6, extended time courses revealed that both STAT1 and STAT3 protein expression were reduced in parallel to almost undetectable levels after 3 hr in response to either cytokine in A2AAR-expressing cells (Figure 3). In contrast, cytokine exposure of GFP-expressing HUVECs under the same conditions produced no significant changes (Figure 3). Thus, a requirement for A2AAR overexpression to trigger down-regulation of STATs is shared by multiple cytokines.

Role of JAK-mediated phosphorylation in targeting STATs for down-regulation

The observed cytokine dependence raised the possibility that JAK-mediated STAT phosphorylation was a critical step responsible for initiating down-regulation. Two approaches were used to test this hypothesis in more detail. First, we assessed the effect of pharmacological inhibition of JAK activity on STAT down-regulation in A2AAR-overexpressing cells. This demonstrated that a concentration of JAK inhibitor sufficient to abolish sIL-6Rα/IL-6-mediated Tyr phosphorylation of both JAK1 and JAK2, as well as the subsequent Tyr705 phosphorylation of STAT3, completely blocked STAT3 down-regulation (Figure 4A,B). Second, HUVECs were co-infected with AVs encoding the A2AAR and either Flag epitope-tagged WT or Tyr705→Phe mutated STAT3, since mutation of Tyr705 renders STAT3 resistant to phosphorylation by JAKs (Kaptein et al., 1996). Under conditions in which WT STAT3 underwent down-regulation in response to sIL-6Rα/IL-6 similar to the effect observed for endogenous STAT3, levels of Tyr705→Phe STAT3 were not altered (Figure 4C). Thus, JAK-mediated Tyr phosphorylation of STATs appears to be essential for promoting their cytokine-mediated down-regulation in A2AAR-overexpressing cells.

Effect of proteasome and lysosome inhibition on STAT down-regulation

Regulated degradation is a frequently used mechanism by which cellular levels of transcription factors, such as p53, are controlled (Watson and Irwin, 2006). To determine whether STAT degradation was the mechanism underlying the effect of A2AAR overexpression, we utilised
the proteasome inhibitor MG132. Pre-incubation with MG132 was found to be sufficient to abolish the effect of the A2AAR on priming STAT3 for down-regulation in response to sIL-6Rα/IL-6 (Figure 5A). Moreover, MG132 abolished the ability of A2AAR overexpression to inhibit sIL-6Rα/IL-6-mediated STAT3 phosphorylation, suggesting that STAT degradation is the mechanism responsible for this effect (Figure 5B).

To examine a role for lysosomal degradation, we tested the effect of preincubation with chloroquine, an inhibitor of lysosomal acidification (van Weert et al., 1995). Preliminary experiments determined that a concentration of 100 μM was effective in blocking vascular endothelial growth factor (VEGF)-induced down-regulation of ubiquitylated VEGF receptor VEGFR2 (data not shown), a process which has been shown to be mediated via lysosomal degradation (Ewan et al., 2006). However chloroquine pretreatment failed to block STAT3 down-regulation in A2AAR-overexpressing cells (Figure 5C). Therefore, STAT down-regulation was sensitive to inhibition of the proteasome but resistant to inhibition of lysosome function, suggesting that the A2AAR specifically targets STATs for proteasomal degradation.

Cytokine-stimulated polyubiquitylation of STATs Proteins targeted for degradation are typically tagged on one or more Lys residues with Lys48-conjugated polyubiquitin chains which are recognised as a degradation signal by the 26S proteasome (Liu et al., 2005; Nalepa et al., 2006). To assess directly whether STATs were ubiquitylated in A2AAR-overexpressing HUVECs, cells were treated with sIL-6Rα/IL-6 and MG132, which allows for accumulation of ubiquitylated proteins by inhibiting proteasome activity. Endogenous STAT3 was immunoprecipitated following denaturing cell lysis to remove any non-covalently associated STAT-binding proteins and inactivate deubiquitinase enzymes. Immunoblotting of STAT3 immunoprecipitates with anti-ubiquitin antibody revealed that sIL-6Rα/IL-6 treatment resulted in the accumulation of a smear of high molecular weight ubiquitylated species only
in A2AAR-overexpressing cells (Figure 6A). Thus, expression of the A2AAR is required to observe cytokine-stimulated ubiquitylation of STAT3 in response to sIL-6Rα/IL-6.

Since down-regulation of STAT3 requires its JAK-mediated phosphorylation on Tyr705 (Figure 4), we then examined the relationship between STAT3 phosphorylation and ubiquitylation in A2AAR-overexpressing cells. To achieve this, Flag-tagged WT and Tyr705→Phe mutated STAT3 were co-expressed in HUVECs with the A2AAR and immunoprecipitated with anti-Flag antibody following denaturing cell lysis after treatment with sIL-6Rα/IL-6 and MG132. This demonstrated that under conditions in which recombinant WT STAT3 is polyubiquitylated similarly to the endogenous protein in response to sIL-6Rα/IL-6 (Figure 6A), no ubiquitylation of the Tyr705→Phe mutant could be detected despite the presence of both WT and Tyr705→Phe STAT3 proteins in immunoprecipitates (Figure 6B).

Together with the data presented in Figure 3, these observations are consistent with a model in which A2AAR overexpression specifically primes JAK-phosphorylated STATs for polyubiquitylation and this triggers their subsequent degradation by the proteasome.

Effect of A2AAR expression on regulation of VEGFR2 expression To be considered functionally significant, the ability of cytokines to promote the accumulation of STAT target gene products should be compromised in A2AAR-overexpressing cells. In the course of our studies, we identified VEGFR2 as a protein in ECs whose levels are positively controlled by STAT3. Thus, incubation of HUVECs with sIL-6Rα/IL-6 increased VEGFR2 expression by 6.8±1.1-fold over untreated controls (p<0.05, n=4), an event which was markedly inhibited by pre-incubation with a maximally effective concentration of JAK inhibitor (Figure 7A). In addition, the sIL-6Rα/IL-6-mediated induction of VEGFR2 could be mimicked by transient overexpression of WT STAT3 and abolished by expression of dominant-negative Tyr705→Phe STAT3 (Kaptein et al., 1996) (Figure 7B). Interestingly, overexpression of the
A2_AAR also increased VEGFR2 expression versus AV.GFP control cells, although this phenomenon appears to be STAT-independent since receptor expression alone produces no detectable changes in STAT phosphorylation (e.g. Figure 1). However, subsequent incubation of A2_AAR-overexpressing cells with sIL-6Rα/IL-6 for 4 hr triggered a 91±6% down regulation of VEGFR2 compared to levels in untreated controls (\(p<0.05\), n=4, Figure 7C).

**Effect of endogenous A2_AARs on STAT protein levels** To assess any potential relevance of the mechanisms we observed for overexpressed A2_AARs on endogenous A2_AAR function, we employed two distinct approaches. Firstly, we assessed the effects of prolonged incubation with A2_AAR-selective inverse agonist ZM241385 on STAT3 protein levels, as sustained (24 hr or longer) treatment with inverse agonists has been shown to unmask effects of low level constitutive activation of endogenous 7TM receptors (Berg et al., 1999). In contrast to the lack of any effect of co-incubation with ZM241385 observed in control HUVECs (Figure 2A), these experiments demonstrated that prolonged pre-incubation of HUVECs with ZM241385 for 24 hr was able to produce a significant increase in STAT3 protein levels which was associated with an increase in sIL-6Rα/IL-6-stimulated STAT3 phosphorylation on Tyr705 (Figure 8A). Similar results were observed if the incubation time was further extended to 48hr (data not shown). Secondly, we utilised mice in which both copies of the A2_AAR gene had been deleted (Ledent et al., 1997). Consistent with other studies utilising this model (Ohta and Sitkovsky, 2001), the absence of A2_AARs potentiated the pro-inflammatory effect of LPS administration, as determined by the significantly elevated serum levels of multiple pro-inflammatory cytokines, such as IL-1, IL-6, TNFα and G-CSF, detectable in LPS-treated A2_AAR KO animals versus WT controls (data not shown). Analysis of STAT expression and phosphorylation in aortic extracts from treated animals revealed that LPS administration significantly increased levels of Tyr701-phosphorylated...
STAT1 and Tyr705-phosphorylated STAT3 in both WT and A2AAR KO animals versus vehicle controls. However, total STAT1 expression and its phosphorylation on Tyr701 were both significantly enhanced in A2AAR KO animals versus WT. Interestingly, this effect was restricted to STAT1, as total and Tyr705-phosphorylated STAT3 levels were not significantly different between WT and A2AAR KO animals (Figure 8B), potentially reflecting a dominance of STAT1-mobilising stimuli at this time point.
DISCUSSION

The A2AAR has been identified as a protective anti-inflammatory 7TM receptor protein not only from pharmacological studies (for example McPherson et al., 2001; Lappas et al., 2005) but also from several studies characterising changes in the inflammatory response in mice in which both copies of the A2AAR gene have been deleted (Ohta and Sitkovsky, 2001; Haskó and Pacher, 2008). Gene dosage studies have provided evidence to show that, at least in T-lymphocytes, there is no A2AAR reserve (Armstrong et al., 2001). Consequently, pathophysiological conditions that alter A2AAR expression, such as EC exposure to Th1 cytokines (Nguyen et al., 2003) or hypoxia (Kobayashi and Millhorn, 1999), are likely to alter cellular responsiveness to inflammatory stimuli. Multiple mechanisms have been proposed to account for its potent anti-inflammatory properties across different cell types; these include inhibition of degranulation and superoxide release from neutrophils, suppression of IL-12 and TNFα release and increased IL-10 production from monocytes and macrophages and the ability of the receptor to suppress activation of pro-inflammatory p38 and NF-κB signalling pathways (Haskó et al., 2007). From the results of this study, we have extended these observations by demonstrating that the A2AAR overexpression can prime cytokine-activated STATs for polyubiquitylation and subsequent degradation by the proteasome, a mechanism which may explain the elevated levels of STAT1 protein observed upon A2AAR depletion in vivo and the elevated levels of STAT3 seen upon sustained incubation of endothelial cells in vitro with A2AAR-selective inverse agonist ZM241385.

While the process described here represents a new mechanism by which 7TM receptors can negatively regulate the JAK/STAT pathway, several studies have already shown that cytokines utilising the JAK/STAT pathway can be regulated by distinct A2AR subtypes. For example, Xu et al. (2008) have observed that the A2BAR is an important repressor of IFNγ-
mediated induction of major histocompatibility complex II transactivator (CIITA) in aortic smooth muscle cells. The sensitivity of the SOCS-3 gene to induction via a cAMP-stimulated pathway involving “exchange protein directly activated by cyclic AMP 1” (Epac1) and C/EBP transcription factors also represents a level of cross-talk between A$_2$AR-activated signalling pathways and the inhibition of ERK1,2 and JAK/STAT signaling from defined suppressor of cytokine signalling-3-regulated cytokine receptors (Sands et al., 2006; Yarwood et al., 2008). Such a mechanism may also explain the ability of A$_2$AAR activation to induce IL-10 in a C/EBP-dependent manner (Csóka et al., 2007).

Similar to the effect we observed on suppression of NF-$\kappa$B in two separate cell systems (Sands et al., 2004), overexpression of the A$_2$AAR was sufficient to prime STATs for degradation in the absence of any exogenous agonist. It is possible that over the course of the experiments, endogenous adenosine released by HUVECs in vitro reaches extracellular levels sufficient to cause A$_2$AAR activation. Endothelial cells are an abundant source of adenosine in vivo due to expression of CD39, an ecto-apyrase that catalyses ATP hydrolysis, and CD73, an ecto-5'-nucleotidase which converts the resulting AMP to adenosine (Zernecke et al., 2006). The accumulation of adenosine that ensues in large blood vessels is thought to play an important protective role in vivo by limiting endothelial cell activation and subsequent monocyte attachment (Zernecke et al., 2006). However, it is also possible that the receptor may be sufficiently active in the absence of agonist to trigger its associated signaling pathways. This phenomenon has been described not only upon overexpression of many 7TM receptors, but also for endogenously expressed receptors as well as virally-encoded 7TM receptors such as Kaposi’s sarcoma-associated herpes virus ORF74 (Vischer et al., 2006). Reversal of the effect of receptor overexpression on STAT down-regulation in a concentration-dependent manner by inverse agonist ZM241385 (Figure 2A,B), coupled with the lack of any significant effect of co-incubation with ADA (Figure 2C), would suggest that the receptor’s constitutive activity is mainly responsible. Interestingly, the reported effect of
A$_{2B}$AR deletion in enhancing IFN$\gamma$-mediated induction of CIITA in vitro (Xu et al., 2008) would tend to suggest that constitutive signalling is not restricted to the A$_{2A}$AR subtype. Despite its obvious importance in controlling the expression and function of various transcription factors such as p53 (Watson and Irwin, 2006) and NF-$\kappa$B (Chen, 2005), relatively few reports have examined STAT degradation. Indeed, while the ability of V proteins encoded by paramyxoviruses to function as STAT E3 ubiquitin ligases is a well-established mechanism by which they subvert the interferon response (Horvath, 2004), native cellular mechanisms controlling STAT degradation are rather less well defined. The first description of STAT degradation centred around the observation that proteasome inhibition produced a more robust accumulation of tyrosine-phosphorylated STAT1 in HeLa cells following exposure to IFN$\gamma$, suggesting that proteasomal degradation is an important mechanism by which STAT1 function is turned off in these cells (Kim and Maniatis, 1996). Subsequently, it was shown that IL-3 exposure results in a time-dependent proteasomal degradation of STAT5A but not STAT1, 2 or 3 in 32D myeloid cells (Wang et al, 2000), although nuclear translocation rather than tyrosine phosphorylation per se seems to be required for degradation to occur (Chen et al., 2006). Another study has shown that the degradation of STAT3 in H4IIE hepatoma cells could be triggered by hyperosmotic stress and occurred independently of phosphorylation on Tyr705 (Lornejad-Schafer et al., 2005). Taken as a whole, none of these observations are consistent with a single unifying mechanism. Thus, while the recent identification of the protein “SLIM” (STAT-interacting protein with LIM domain) as an E3 ubiquitin ligase able to trigger the polyubiquitylation of STAT1 and STAT4 is an important advance (Tanaka et al., 2005; Gao et al., 2007), it is unlikely to account for all the STAT degradation phenomena reported in the literature. Related to this issue, we found that inhibition of proteasome function was sufficient to block the inhibitory effect of the A$_{2A}$AR on STAT3 phosphorylation, demonstrating that priming of
STATs for degradation is the only mechanism responsible for the reduced cytokine-stimulated STAT phosphorylation observed in A$_{2A}$AR-overexpressing cells. This contrasts with the observation that exogenous expression of SLIM in HEK293 cells was able to inhibit cytokine-stimulated tyrosine phosphorylation of STAT4 as well as promoting its polyubiquitylation and degradation by the proteasome (Tanaka et al., 2005). This might suggest the involvement of another E3 ubiquitin ligase in HUVECs, and consistent with this hypothesis we have been unable to detect SLIM message or protein in HUVECs following cytokine stimulation of A$_{2A}$AR-overexpressing cells (Sahfi MMA, Sands WA, Palmer TM, unpublished observations). In addition, while Tyr phosphorylation is clearly the critical step in targeting STATs for degradation in A$_{2A}$AR-overexpressing cells, it is unclear as to whether it functions simply as a classical phosphodegron, or whether the nuclear translocation that occurs as a result of phosphorylation is also important for localising the phosphorylated STAT dimer with the relevant E3 ubiquitin ligase.

More generally, the identification in this study of a new mechanism by which cytokine signaling can be turned off by targeting tyrosine-phosphorylated STATs for degradation has significant implications for diseases associated with altered regulation of the JAK/STAT pathway. It also reinforces the argument that potentiation of A$_{2A}$AR function might prove a useful strategy with which to down-regulate pro-inflammatory responses by virtue of its capacity to inhibit multiple pro-inflammatory processes utilised by distinct stimuli. However, it also raises the issue that any beneficial effects of A$_{2A}$AR-selective inverse agonists in neurodegenerative conditions such as Huntington’s and Parkinson’s diseases (Jenner et al., 2009) might also have undesirable pro-inflammatory side-effects.
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REFERENCES


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FIGURE LEGENDS

**Figure 1** Effect of A2AAR overexpression on STAT3 phosphorylation and expression in response to sIL-6Rα/IL-6

*Panel A:* HUVECs were infected with the indicated AVs at an MOI of 25 as described in the Materials and Methods prior to stimulation with 5 μM CGS21680 (CGS) for the indicated times and preparation of soluble cell extracts for SDS-PAGE and immunoblotting with anti-Thr202/Tyr204 phospho-ERK1,2 and anti-myc 9E10 antibodies to identify recombinant A2AAR.

*Panel B:* HUVECs were infected with recombinant AVs as in Panel A prior to treatment with or without 25 ng/ml sIL-6Rα/5 ng/ml IL-6 (sIL-6Rα/IL-6) for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total and Tyr705 phospho-STAT3 levels from three experiments is presented (*p<0.05 versus the response observed in AV.GFP-infected cells).

**Figure 2** Pharmacology of the A2AAR effect on STAT3 phosphorylation and expression

*Panel A:* HUVECs were infected with recombinant AVs prior to treatment with or without sIL-6Rα/IL-6 in the presence of A2AAR-selective agonist CGS21680 (5 μM) or selective inverse agonist ZM241385 (1 μM) for 1 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A2AAR-expressing cells from three such experiments is presented (*p<0.05 versus levels in AV.mycA2AAR-infected vehicle-treated cells).
Panel B: HUVECs were infected with recombinant AVs as in Panel A prior to treatment with or without sIL-6Rα/IL-6 for 3 hr in the presence of increasing concentrations of ZM241385 as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of the recovery of total STAT3 levels in A2AAR-expressing cells from three such experiments is presented.

Panel C: HUVECs were infected with recombinant AVs as in Panel A prior to pre-treatment with or without 3U/ml adenosine deaminase (ADA) for 3 hr followed by treatment with or without sIL-6Rα/IL-6 for 1 hr. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A2AAR-expressing cells from three such experiments is presented (*p<0.05 versus levels in vehicle-treated cells).

Figure 3  The effect of A2AAR overexpression on IFNα regulation of STAT1 and STAT3
HUVECs were infected with recombinant AVs prior to treatment with or without 500 U/ml IFNα or sIL-6Rα/IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-STAT1, STAT3 and GAPDH antibodies. Quantitative analysis of total STAT3 levels from three experiments with each cytokine is presented (*p<0.05 versus the levels in AV.GFP-infected cells at the given time point).

Figure 4  A role for JAK phosphorylation in triggering cytokine-mediated STAT down-regulation in A2AAR-overexpressing cells
Panel A: HUVECs were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without sIL-6Rα/IL-6 for 30 min as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with total and anti-Tyr1022/1023 phospho-JAK1, Tyr1007/1008 phospho-JAK2, and Tyr705 phospho-STAT3 antibodies as indicated.

Panel B: HUVECs were infected with the indicated AVs prior to pre-treatment with or without 0.1 μM JAK inhibitor I for 30 min and treatment with sIL-6Rα/IL-6 for up to 3 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A2AAR-expressing cells from three experiments is presented (*p<0.05 versus STAT levels in vehicle-pretreated cells at the given time point).

Panel C: HUVECs were co-infected with AV.myc-A2AAR and either AV.Flag-WT STAT3, AV.Flag-Tyr705→Phe STAT3 or AV.GFP (control) prior to treatment with or without sIL-6Rα/IL-6 for 1 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with M5 anti-Flag and GAPDH antibodies. Quantitative analysis of total Flag-STAT3 levels from three experiments is presented (*p<0.05 versus levels in vehicle-treated cells).

Figure 5 Effect of proteasome and lysosome inhibitors on sIL-6Rα/IL-6-induced STAT3 phosphorylation and down-regulation in A2AAR-overexpressing HUVECs

Panel A: HUVECs were infected with the indicated AVs prior to pre-treatment with or without 6 μM MG132 for 30 min and treatment with sIL-6Rα/IL-6 for up to 3 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total...
STAT3 levels in A2AAR-expressing cells from three experiments is presented (*p<0.05 versus STAT3 levels in vehicle-pretreated cells at the given time point).

Panel B: HUVECs were infected with the indicated AVs prior to pre-treatment with MG132 and sIL-6Rα/IL-6 as in Panel A. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of Tyr705 phospho-STAT3 levels from three experiments is presented.

Panel C: HUVECs were infected with the indicated AVs prior to pre-treatment with or without 100 μM chloroquine for 30 min and treatment with sIL-6Rα/IL-6 for 1 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. This is one of three experiments that produced similar results.

Figure 6 Effect of A2AAR overexpression on STAT ubiquitylation in response to cytokine stimulation

Panel A: HUVECs were infected with the indicated AVs prior to pre-treatment with 6 μM MG132 for 30 min and incubation with or without sIL-6Rα/IL-6 for 1 hr as indicated. Samples were then denatured by heating in SDS-containing buffer prior to dilution into excess non-ionic detergent for preparation of clarified extracts, equalization of protein content and immunoprecipitation of STAT3. Immunoprecipitates were fractionated by SDS-PAGE for immunoblotting with anti-ubiquitin and STAT3 antibodies.

Panel B: HUVECs were co-infected with AV.myc-A2AAR and either AV.GFP (control), AV.Flag-WT STAT3 or AV.Flag-Tyr705→Phe STAT3 prior to treatment with MG132 and incubation with or without sIL-6Rα/IL-6 for 1 hr as indicated. Clarified extracts were then prepared for immunoprecipitation of Flag-STAT3 proteins using M2 antibody-coupled
Seharose beads. Samples were then fractionated by SDS-PAGE prior to immunoblotting with anti-ubiquitin and STAT3 antibodies.

**Figure 7** Effect of A2AAR overexpression on STAT3-regulated induction of VEGFR2 by sIL-6Rα/IL-6

*Panel A:* HUVECs were pre-incubated with or without 0.1 μM JAK inhibitor I for 30 min prior to treatment with or without sIL-6Rα/IL-6 for the indicated times. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2 and GAPDH antibodies.

*Panel B:* HUVECs were infected with AV.GFP (control), AV.Flag-WT STAT3 or AV.Flag-Tyr705→Phe STAT3 prior to treatment with or without sIL-6Rα/IL-6 for 4 hrs. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2, anti-Flag M5 and GAPDH antibodies.

*Panel C:* HUVECs were infected with the indicated AVs prior to treatment with sIL-6Rα/IL-6 for 2 or 4 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2 and GAPDH antibodies.

**Figure 8** Effect of endogenous A2AARs on STAT phosphorylation and expression

*Panel A:* HUVECs were pretreated with either vehicle or A2AAR-selective inverse agonist ZM241385 (1 μM) for 24 hr as indicated prior to further incubation with or without sIL-6Rα/IL-6 for 1 hr as indicated. Soluble cell extracts equalised for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels from three experiments is presented (*p<0.05 versus STAT3 levels in cells treated with vehicle alone).
Panel B: A2AAR\textsuperscript{+/+} (WT) and homozygous A2AAR\textsuperscript{-/-} KO mice were injected with either PBS vehicle or 0.4 mg/kg LPS for 4 hr prior to isolation of aortae for solubilisation in sample buffer, SDS-PAGE and immunoblot analysis of total and Tyr-phosphorylated STATs1 and 3 as indicated. Quantitative analysis of 8 animals/group is shown (STAT1 and 3 phosphorylation graphs; *\(p<0.001\) versus vehicle-treated animals, *\(p<0.001\) versus LPS-treated WT animals; STAT expression graph; *\(p<0.05\) versus WT animals).
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B

Incubation time (min) + sIL-6Rα/IL-6

Mr (kDa) 0 15 30 60 0 15 30 60

Phospho-STAT3

Total STAT3

GAPDH

AV.GFP AV.mycA2AR

Tyr705 STAT3 Phosphorylation (% of maximal response)

sIL-6Rα/IL-6 Exposure Time (min)

GFP mycA2AR

Total STAT3 levels (% of untreated control)

sIL-6Rα/IL-6 Exposure Time (min)

GFP mycA2AR
A

- - - + - - - + : ZM241385, 1 hr
- - + - - + + : CGS21680, 1 hr
- + + + + + : sIL-6Rα/IL-6, 1 hr

Mr (kDa)
97
97
37

Phospho-STAT3
Total STAT3
GAPDH

AV.GFP AV.mycA2AAR

Total STAT3 levels (% of untreated GFP control)

- + + + - - + : sIL-Rα/IL-6, 1 hr
: Pretreatments, 1 hr

CGS ZM CGS ZM

AV.GFP AV.mycA2AAR

2A
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Total STAT3 levels (% of untreated control)

IFN\(\alpha\) Exposure Time (Hr)

Total STAT3 levels (% of untreated control)

sIL-6R\(\alpha\)/IL-6 Exposure Time (Hr)

Mr (kDa) 0 1 2 3 0 1 2 3 Incubation time (hr) + IFN\(\alpha\) + sIL-6R\(\alpha\)/IL-6 Mr (kDa) 0 1 2 3 0 1 2 3 Incubation time (hr) + IFN\(\alpha\) + sIL-6R\(\alpha\)/IL-6

GFP mycA\(_{2\alpha}\)AR

AV.GFP AV.mycA\(_{2\alpha}\)AR

GFP

mycA\(_{2\alpha}\)AR
B

**Figure B**

**Graph:**
- **X-axis:** sIL-6Rα/IL-6 Exposure Time (Hr)
- **Y-axis:** Total STAT3 levels (% of untreated control)
- **Legend:**
  - Vehicle
  - + JAK Inhibitor

**Notes:**
- **AV.GFP** and **AV.mycA2xAR**
- **Incubation time (hr)**
- **+0.1 μM JAK inhibitor**
- **30 min pre-incubation**

**Immunoblot:**
- **Total STAT3**
- **GAPDH**
Total Flag-STAT3
(% untreated control, set at 100)

Tyr705Phe
- +

WT
- +

:: sIL-6Rα/IL-6, 1 hr

Mr (kDa)

37 67

GFP control

:: sIL-6Rα/IL-6, 1 hr

Flag-STAT3

WT STAT3

:: Flag-STAT3

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**Incubation time (hr)**

+siL-6Rα/IL-6

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**AV.GFP**

**AV.myc2AAR**

**Total STAT3**

**GAPDH**

+6 μM MG132

30 min pre-incubation

**Total STAT3 levels**

(% of untreated control)

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**Incubation time (hr)**

+ sIL-6Rα/IL-6

**Phospho-STAT3**

**Total STAT3**

+6 μM MG132

30 min pre-incubation

**Tyr705 STAT3 phosphorylation (Fold stim over basal)**

**sIL-6Rα/IL-6 Exposure Time (hr)**

- AV.GFP
- AV.mycA2AAR
C

- + - +

: 100 μM chloroquine
30 min preincubation

- - + +

: sIL-6Rα/IL-6, 1hr

AV.GFP AV.mycA2AAR

Total STAT3

GAPDH
A

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: +sIL-6Rα/IL-6 (hr)

- VEGFR2
- GAPDH

: 0.1 μM JAK Inhibitor I
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Mr (kDa)  
- 220  
- 97  
- 37

- VEGFR2
- Flag-STAT3
- GAPDH