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Th1 Cytokines Regulate Adenosine Receptors and Their Downstream Signaling Elements in Human Microvascular Endothelial Cells[1]

Nguyen D. Khoa, M. Carmen Montesinos, Adrienne J. Williams, Maureen Kelly, and Bruce N. Cronstein[2]

We and others have shown that adenosine, acting at its receptors, is a potent modulator of inflammation and angiogenesis. To better understand the regulation of adenosine receptors during these processes we studied the effects of IL-1, TNF-α, and IFN-γ on expression and function of adenosine receptors and select members of their coupling G proteins in human dermal microvascular endothelial cells (HMVEC). HMVEC expressed message and protein for A_{2A} and A_{2B}, but not A_1 or A_3 receptors. IL-1 and TNF-α treatment increased message and protein expression of A_{2A} and A_{2B} receptor. IFN-γ treatment also increased the expression of A_{2B} receptors, but decreased expression of A_{2A} receptors. Resting HMVEC and IFN-γ-treated cells showed minimal cAMP response to the selective A_{2A} receptor agonist 2-[2-(4-chlorophenyl)ethoxy]adenosine (MRE0094). In contrast, MRE0094 stimulated a dose-dependent increase in cAMP levels in TNF-α-treated cells that was almost completely blocked by the A_{2A} receptor antagonist ZM-241385 (4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol). The nonselective adenosine receptor agonist 5′-(*N*-ethylcarboxamido)adenosine increased cAMP levels in both TNF-α- and IFN-γ-treated cells, but not control cells, and its effect was only partially reversed by ZM-241385 in TNF-α-treated cells and not affected in IFN-γ-treated cells. HMVEC expressed a higher level of G protein β1 isoform than β4 isoform. Although none of the cytokines tested affected $G_{\beta 1}$ expression, both IL-1 and TNF-α significantly up-regulated $G_{\beta 4}$ expression. These findings indicate that inflammatory cytokines modulate adenosine receptor expression and function on HMVECs and suggest that the interaction between proinflammatory cytokines and adenosine receptors may affect therapeutic responses to anti-inflammatory drugs that act via adenosine-dependent mechanisms. ***The Journal of Immunology*****, 2003, 171: 3991–3998.**

Adenosine, acting at one or more of its receptors, is a potent endogenous regulator of inflammation that inhibits the proinflammatory actions of neutrophils, monocytes, lymphocytes, and endothelial cells (see review in Ref. 1). Endothelial cells are now believed to play a more active role in the inflammatory response than previously thought. The action of adenosine and its receptors on endothelial cell functions is of growing interest. Accumulating evidence indicates that, along with other effects, adenosine may modulate the response of endothelial cells to inflammatory stimuli during the development of inflammatory lesions. For example, adenosine suppresses TNF-α-stimulated expression of tissue factor, a principal initiator of coagulation, in endothelial cells (2). In addition, adenosine, acting via A_{2A} receptors, diminishes endothelial secretion of the inflammatory cytokines IL-6 and IL-8 (3), whereas the selective occupancy of the A_{2B} receptor stimulates IL-8 production in a human microvascular endothelial cell line (4). Moreover, both A_{2A} and A_{2B} adenosine receptors are reported to mediate angiogenesis (4–7), a feature of chronic inflammation.

Endothelial cells have been reported to express several or all of the four known adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) depending on the method used to detect expression and, more importantly, the source of the endothelial cells (4, 8–10). Nevertheless, regulation of adenosine receptor expression and function in endothelial cells in general, and particularly in cells derived from the microvasculature, which are more directly involved in angiogenesis and inflammatory reactions than other endothelial cells, remains obscure.

In some cell types there is clear evidence that the expression and function of adenosine receptors are regulated by adenosine receptor agonists or antagonists (11–13), glucocorticoid hormones (14, 15), and growth factors (16, 17). More interestingly, Xaus and colleagues (18) reported that IFN-γ, one of the most critical cytokines in acute and chronic inflammation, up-regulates the expression of A_{2B} receptors in murine macrophages and promotes macrophage activation. We first demonstrated that IL-1, TNF-α, and IFN-γ, the most prominent Th1 inflammatory cytokines, regulate the function and expression of adenosine A_{2A} and A_{2B} receptors in the human monocytoid cell line THP-1 (19). More recently, Trincavelli and colleagues (20) reported similar up-regulation of the A_{2A} receptor in rat PC12 cells by proinflammatory cytokines IL-1 and TNF-α.

In the present study we investigated the effects of Th1 cytokines (IL-1, TNF-α, and IFN-γ) on the expression and function of adenosine receptor subtypes in human microvascular endothelial cells to further understand the interaction between inflammatory cytokines and adenosine receptors. In addition, we tested the effects of

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these cytokines on the expression of select downstream signal transduction proteins. We report here that human dermal (foreskin) microvascular endothelial cells express A_{2A} and A_{2B} receptors, but not A_1 and A_3 receptors. More importantly, in these cells the Th1 cytokines differentially modulate the expression and function of A_{2A} and A_{2B} receptors. The expression of $G_{\beta 4}$ protein, but not $G_{\alpha s}$ or $G_{\beta 1}$ protein, was also modulated by IL-1 and TNF-α. Up-regulation of A_{2A} receptor expression and function leads to enhanced vascular endothelial growth factor (VEGF)[3] mRNA expression induced by the A_{2A} selective agonist CGS21680.

Materials and Methods

Reagents

Recombinant human IL-1α, TNF-α, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The selective adenosine A_{2A} receptor agonist 2-[2-(4-chlorophenyl)ethoxy]adenosine (MRE0094) (2) was supplied by King Pharmaceuticals (21), 5′-(*N*-ethylcarboxamido)adenosine (NECA) and 2-{4-[(2-carboxyethyl)phenyl]ethylamino}-5′-*N*-ethylcarboxamidoadenosine (CGS-21680) were purchased from Sigma-Aldrich (St. Louis, MO), and 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol (ZM-241385) was obtained from Tocris Cookson (Ballwin, MO). Murine mAb (clone 7F6-G5-A2) against human A_{2A} receptor was a gift from Dr. J. Linden (University of Virginia School of Medicine, Charlottesville, VA) (22), and goat polyclonal Abs against human A_1, A_{2B}, and A_3 receptors and G proteins and alkaline phosphatase-conjugated secondary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Human dermal microvascular endothelial cells (HMVEC), originally derived from foreskin, were obtained from BioWhittaker (Walkersville, MD). They were cultured in supplemented EGM-2 medium at 37°C in a humidified atmosphere consisting of 5% CO_2. Cells used in all experiments were between passages 3 and 5.

RNA extraction and semiquantitative and real-time RT-PCR

mRNA was isolated from HMVEC using a MicroFastTrack kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA was synthesized, and subsequent PCR was performed using the GeneAmp RNA-PCR Core Kit (PerkinElmer, Branchburg, NJ). The cycle number was adjusted to allow PCR to proceed in a linear range. cDNA samples were subject to serial dilutions before proceeding to PCR for target genes and the control housekeeping gene GAPDH. PCR product was separated by electrophoresis in 2% agarose gel containing ethidium bromide (0.2 μg/ml), visualized with a UV transilluminator, and digitally photographed. The amplicon was quantitated densitometrically using Kodak Digital Science software, and all values were normalized to the GAPDH amplicon, since in preliminary experiments we observed that under the conditions studied, using the same amount of input mRNA for cDNA synthesis and subsequent PCR, the levels of GAPDH message appeared to be very similar with all treatments of HMVEC.

Real-time PCR was performed on cDNA using the Smart Cycler System (Cepheid, Sunnyvale, CA) and reagents from the SYBR-Green PCR kit (PE Applied Biosystems, Foster City, CA). A typical reaction was set up in a total volume of 25 μl using unknown cDNA sample or standard cDNA with a known concentration as a template. The reaction mixture is similar to conventional PCR with the addition of SYBR-Green to the reaction buffer, which allows dsDNA to produce fluorescence signals, resulting in a real-time curve. The PCR run was programmed with software provided with the Smart Cycler System using a protocol specific for the target DNA. The threshold cycle at which amplicon was detectable was used for the purpose of quantitation. Data from the target gene were normalized to GAPDH.

Primers used for RT-PCR (Table I) were designed using mRNA sequences from GenBank to amplify cDNA fragments that cross an intron in the genomic DNA. The sequence of amplicon was determined to confirm the identity of the cDNA amplified. Sequences of primers for human GAPDH have been previously described (19).

[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; CGS-21680, 2-{4-[(2-carboxyethyl)phenyl]ethylamino}-5′-*N*-ethylcarboxamidoadenosine; HMVEC, human microvascular endothelial cells; MRE0094, 2-[2-(4-chlorophenyl)ethoxy]adenosine; NECA, 5′-(*N*-ethylcarboxamido)adenosine; ZM-241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol.

Western blot analysis

Cells were washed in ice-cold PBS and collected using a rubber scraper. Crude membrane protein preparations were obtained following sonication of cells in a modification of a previously described technique (17, 23). Protein concentration was measured by standard protein assay. Equal amounts of protein (20–40 μg/lane) were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Control Ponceau staining was performed after gel transfer to verify that equal amounts of protein were loaded. Nonspecific Ab binding was blocked with 3% nonfat milk in Tris-buffered saline containing 0.3% Tween 20. Subsequently, the blot membranes were incubated with primary Abs for 1–4 h and then with an alkaline phosphatase-conjugated secondary Ab for 1 h. After each Ab incubation, blots were extensively washed with Tris-buffered saline containing 0.3% Tween 20 (three or four times, 5 min each time). Finally, the blots were exposed to ECF substrate (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned using the Storm phosphorimager system. The band intensity was then directly quantitated using ImageQuant software.

Adenosine receptor stimulation and cAMP assay

HMVECs were placed in 24-well plates at a density of 10^5 cells/well and were treated overnight with TNF-α or IFN-γ. On the day of the cAMP assay, the cells were stimulated for 10–15 min with MRE0094 (10^{-8}–10^{-5} M) or NECA (10^{-7}–10^{-5} M) in the presence or the absence of ZM-241385 (10^{-5} M). ZM-241385 was added to the cells 30 min before the addition of the agonist. Culture supernatants were removed, and cells were lysed and assayed for cellular cAMP concentrations using the cAMP enzyme immunoassay system kit (Amersham Pharmacia Biotech) following the manufacturer's instructions. A time-course study was performed with 1 μM MRE0094 stimulation to detect a time at which the optimal response to the agonist was observed.

Analysis of VEGF mRNA expression

HMVECs were pretreated overnight with TNF-α or IFN-γ or both TNF-α and IFN-γ, then stimulated with CGS-21680 (1 μM) for 4 h in the presence or the absence of 1 μM ZM-241385. mRNA was isolated, and the expression of VEGF was examined by quantitative real-time PCR as described.

Data analysis

All statistical analysis was performed using the SigmaStat program on a Pentium 3-based computer. Data are presented as the mean ± SEM. One- and two-way ANOVAs were used to determine statistical significance between means or curves. Differences with $p < 0.05$ were considered significant.

Results

Expression of adenosine receptors in HMVEC

The results of RT-PCR and Western blot analysis showed the expression of A_{2A} and A_{2B} receptors in both resting and cytokine-treated HMVECs (Fig. 1-4). However, neither message nor protein for A_1 or A_3 receptors was detected in those cells (data not shown). The same primers and Abs were able to detect the expression of A_1 and A_3 receptors in primary human dermal fibroblasts, the human monocytoid cell line THP-1, and HUVEC (9) (data not shown), confirming the absence of mRNA for A_1 and A_3 expression in nontreated and treated HMVECs.

Effects of IL-1, TNF-α, and IFN-γ on A_{2A} receptor expression

As shown in Fig. 1, treatment of cultured HMVECs with IL-1, TNF-α, or IFN-γ altered the message level of A_{2A} receptor. Semiquantitative RT-PCR showed an increase in the A_{2A} message to 167 ± 17 and 150 ± 15% of the control value after 3-h incubation with IL-1 and TNF-α, respectively ($n = 5$; $p < 0.01$ vs control for IL-1; $p < 0.05$ vs control for TNF-α). In contrast, 3-h treatment with IFN-γ decreased the A_{2A} message to 72 ± 11% of the control value ($n = 5$; $p = 0.055$). The effects of the cytokines on the expression of A_{2A} receptors remained consistent following overnight treatment; the message levels of A_{2A} changed to 165 ± 14, 161 ± 15, and 57 ± 18% of the control value in IL-1-, TNF-α-, and IFN-γ-treated cells, respectively ($n = 5$; $p < 0.01$ vs control for IL-1 and TNF-α; $p < 0.05$ vs control for IFN-γ; Fig. 1*B*).

Table I. *Sequences of primers for human adenosine receptors, G proteins, and VEGF*[a]

Name	Type	Sequence	Length of PCR Product (bp)
A_1	Forward	5′-TCCATCTCAGCTTTCCAGGC-3′	422
	Reverse	5′-CTCGAACTCGCACTTGATCAC-3′	
A_{2A}	Forward	5′-ACCTGCAGAACGTCACCAAC-3′	524
	Reverse	5′-TCTGCTTCAGCTGTCGTCGC-3′	
A_{2B}	Forward	5′-CACAGGACGCGCTGTACGTG-3′	464
	Reverse	5′-TTCTGTGCAGTTGTTGGTGG-3′	
A_3	Forward	5′-AACGTGCTGGTCATCTGCGTGGTC-3′	440
	Reverse	5′-GTAGTCCATTCTCATGACGGAAAC-3′	
$G_{\alpha S}$	Forward	5′-CTGCTCGCTGAGAAAGTCCT-3′	498
	Reverse	5′-GTAGGCCGCCTTAAGCTTCT-3′	
$G_{\beta 1}$	Forward	5′-GCCGAGCAACTTAAGAACCA-3′	436
	Reverse	5′-TGATTGTCATCCAGGAATCG-3′	
$G_{\beta 4}$	Forward	5′-ATGATGCAACGCTTGTTCAG-3′	476
	Reverse	5′-CAGAATGCCCAGTGAATGTG-3′	
VEGF	Forward	5′-CTACCTCCACCATGCCAAGT-3′	344
	Reverse	5′-TGCATTCACATTTGTTGTGC-3′	

[a] All primers were designed based on mRNA sequences published in GenBank, and the identity of the amplicon was confirmed by sequencing. GenBank accession numbers are X68485 (A_1), X68486 (A_{2A}), X68487 (A_{2B}), L22607 (A_3), X07036 ($G_{\alpha S}$), AF501882 ($G_{\beta 1}$), AF300648 ($G_{\beta 4}$), and S79680 (VEGF).

In some experiments, in parallel with semiquantitative RT-PCR, the expression of mRNA for A_{2A} receptor was examined by real-time PCR, which yields a more precise estimate of copy numbers of the target gene. By this analysis, mRNA for A_{2A} receptor was ~0.54 ± 0.14% of GAPDH mRNA in resting HMVECs. Interestingly, the data obtained by real-time PCR not only confirmed the findings by semiquantitative RT-PCR, but also revealed more striking cytokine-induced changes in A_{2A} receptor expression (Fig. 1*C*). Furthermore, real-time PCR also showed that when the cultured cells are exposed to both TNF-α (100 U/ml) and IFN-γ (100 U/ml) together, the TNF-α-mediated increase in the expression of A_{2A} receptor expression dominates the IFN-γ-mediated suppression of A_{2A} receptor expression (Fig. 1*D*; $p < 0.01$ vs control). There was no significant difference between the extent of A_{2A} receptor up-regulation in cells treated with TNF-α alone or with the combination of TNF-α and IFN-γ.

Immunoblotting of membrane preparations using an mAb against A_{2A} receptor detected a clear band of 45 kDa corresponding to the m.w. of the A_{2A} receptor. The changes in A_{2A} receptor protein levels induced by the cytokines paralleled the changes observed in A_{2A} receptor message, although the magnitude of the protein changes was less. Protein levels of A_{2A} receptors were

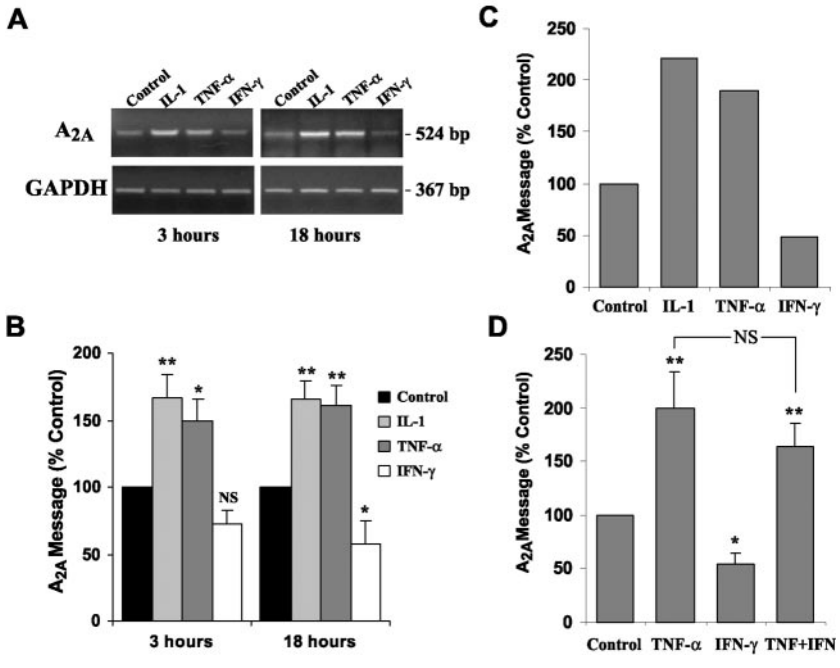

FIGURE 1. Effects of inflammatory cytokines on the expression of adenosine A_{2A} receptor in HMVEC. mRNA was extracted from HMVECs treated with IL-1 (10 U/ml), TNF-α (100 U/ml), IFN-γ (100 U/ml), or medium alone (Control) for 3 h or overnight (18 h), as indicated, and subjected to semiquantitative RT-PCR and real-time PCR for A_{2A} receptor. *A*, Representative agarose gel electrophoresis of RT-PCR for the A_{2A} receptor. *B*, Semiquantitative RT-PCR for the A_{2A} receptor message. PCRs were performed with serially diluted reverse transcriptase, and the level of A_{2A} receptor cDNA in PCR product was densitometrically quantitated and normalized to GAPDH. Data shown are the mean ± SEM percentages of control from five independent experiments. *C*, Quantitative real-time PCR for the A_{2A} receptor message. Copy number of A_{2A} receptor message in cDNA samples from treated HMVEC was estimated by real-time PCR using accompanying standard DNA templates with known concentrations. Data were normalized to GAPDH amplicon and are shown as the average percentage of control from two experiments. *D*, Real-time PCR analysis of A_{2A} receptor expression in HMVECs treated overnight with TNF-α, IFN-γ, or both TNF-α and IFN-γ. Data shown were derived from three experiments separately from experiments shown in *C*. *, $p < 0.05$; **, $p < 0.01$ (vs control).

increased by IL-1 and TNF-α to 129 $\pm$ 7 and 134 $\pm$ 6% of the control, respectively, and were decreased by IFN-γ to 70 $\pm$ 4% of the control ($n = 4$; $p < 0.05$ vs control for IL-1; $p < 0.01$vs control for TNF-α and IFN-γ; Fig. 2).

Effects of inflammatory cytokines on A_{2B} receptor expression

As shown in Fig. 3, we observed up-regulation of the A_{2B} receptor message in HMVEC following treatment with IL-1, TNF-α, or IFN-γ. However, the change in A_{2B} message detected by semiquantitative RT-PCR was significant only after overnight incubation, but not following 3-h incubation. Overnight treatment with IL-1, TNF-α, or IFN-γ increased the message for A_{2B} receptors to 136 $\pm$ 15, 131 $\pm$ 14, and 140 $\pm$ 12% of the control value, respectively ($n = 5$; $p < 0.05$ vs control for all; Fig. 3*B*).

The expression of A_{2B} receptor message in HMVEC following overnight cytokine treatment was further examined by real-time PCR. This confirmed a cytokine-induced up-regulation of A_{2B} receptor, which was similar in magnitude to that detected by semiquantitative RT-PCR (data not shown). As estimated by real-time PCR, the message level for A_{2B} receptor in resting HMVEC was 0.89 $\pm$ 0.12% of GAPDH mRNA ($n = 3$), ~1.6-fold higher than the A_{2A} receptor level in the same cells.

Immunoblot of crude membrane preparations using a polyclonal Ab against A_{2B} receptor detected a major band of 35–36 kDa, the known m.w. for the A_{2B} receptor protein (Fig. 4*A*). Consistent with the changes in A_{2B} message, IL-1, TNF-α, and IFN-γ increased the A_{2B} protein level by 125 $\pm$ 6, 141 $\pm$ 10, and 131 $\pm$ 8% of the control value, respectively ($n = 3$; $p < 0.05$ vs control for all; Fig. 4*B*).

Functional activation of A_2 receptors in cytokine-treated HMVEC

As demonstrated above, HMVECs express both A_{2A} and A_{2B} receptors. Those two receptor subtypes are generally known to signal via the $G_{\alpha s}$-adenylyl cyclase-cAMP system. To determine whether the changes in the expression of A_{2A} and A_{2B} receptors lead to relevant functional changes, we studied the cAMP response following agonist stimulation in HMVEC treated with TNF-α or IFN-γ. The basal cAMP level in TNF-α-treated cells was lower than that in untreated control cells, whereas IFN-γ-treated cells had a slightly higher level of basal cAMP compared with controls. These differences were not, however, statistically significant (Fig.

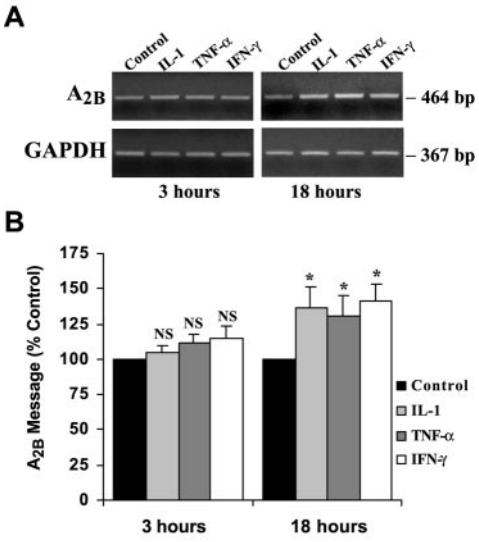

FIGURE 3. Effects of inflammatory cytokines on the expression of adenosine A_{2B} receptor in HMVEC. Semiquantitative RT-PCR was performed on mRNA isolated from HMVEC treated with cytokines as described. *A*, Representative agarose gel of electrophoresed RT-PCR products. *B*, Expression levels of A_{2B} receptors were quantitated after GAPDH normalization. Data shown are the mean $\pm$ SEM percentages of control from five independent experiments. $*$, $p < 0.05$.

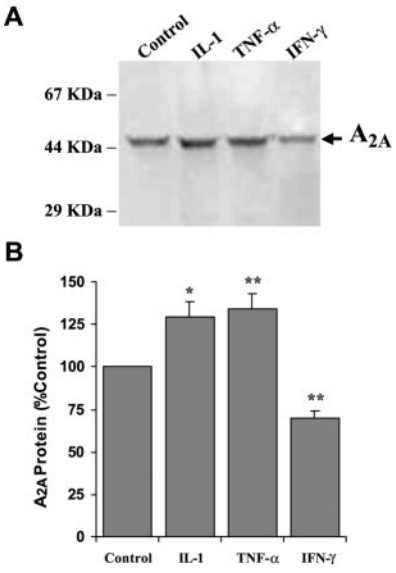

FIGURE 2. Western blot analysis of adenosine A_{2A} receptors in HMVEC treated overnight with IL-1, TNF-α, or IFN-γ. Equal amounts (20 μg in each lane) of crude membrane proteins isolated from treated cells were separated by 10% SDS-PAGE. A_{2A} receptor expression was assessed by Western blot using a murine mAb to the A_{2A} receptor as described. *A*, Representative Western blot for A_{2A} receptor. *B*, Quantitation of A_{2A} receptor protein expression in treated cells as assessed by densitometric analysis of Western blots. Data are expressed as the mean ($\pm$SEM) percentage of control ($n = 4$). $*$, $p < 0.05$; $**$, $p < 0.01$ (vs control).

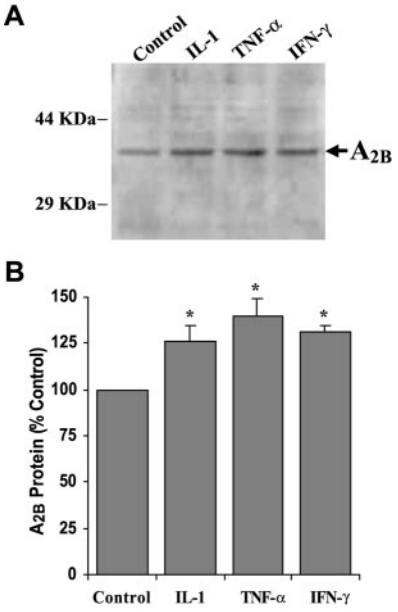

FIGURE 4. Western blot analysis of adenosine A_{2B} receptor expression in HMVEC following overnight cytokine treatment. Crude membrane protein from cytokine-treated HMVEC (30 μg in each lane) was subjected to Western blot using a polyclonal Ab against adenosine A_{2B} receptors. *A*, Representative Western blots of A_{2B} receptors. *B*, Semiquantitation of A_{2B} receptor protein expression. Shown are the mean $\pm$ SEM percentages of control ($n = 3$). $*$, $p < 0.05$.

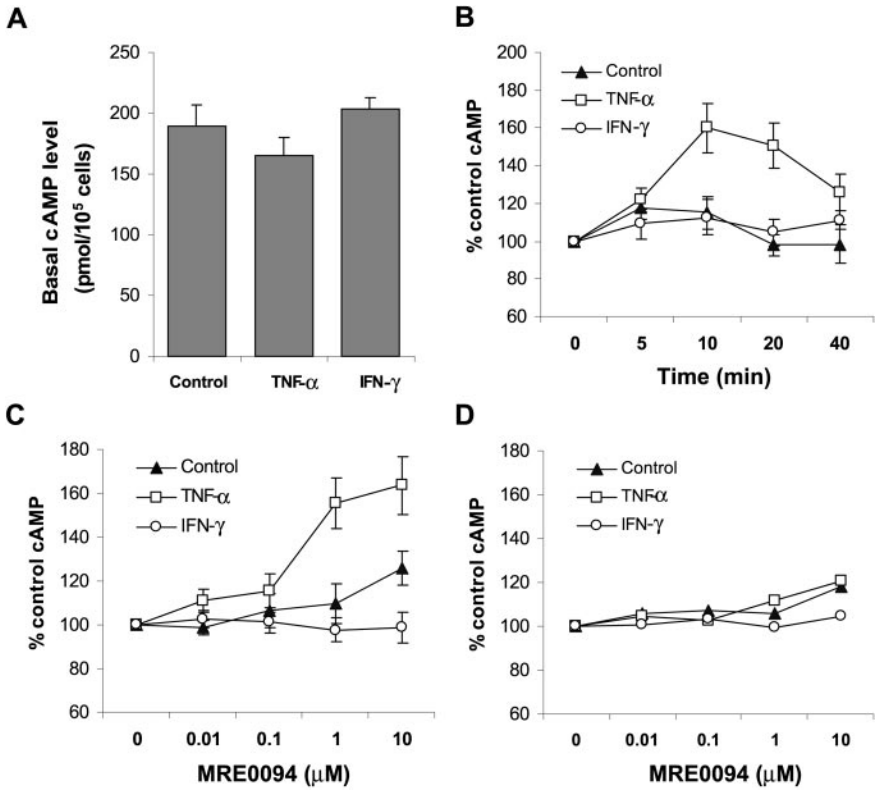

FIGURE 5. cAMP accumulation by the A_{2A} adenosine receptor agonist MRE0094 in cytokine-treated HMVEC. HMVECs were treated overnight with TNF-α or IFN-γ and thereafter stimulated with MRE0094 as described. The cellular cAMP content of treated HMVEC was measured by ELISA. *A*, Basal cAMP levels in cytokine-treated HMVEC (without agonist stimulation). *B*, Cytokine-pretreated HMVECs were stimulated with 1 μM MRE0094 for various times as indicated. *C*, Cytokine-treated HMVECs were stimulated for 10 min with the indicated concentrations of MRE0094. *D*, Enhanced cAMP accumulation in TNF-α-treated HMVEC by the agonist MRE0094 was attenuated in the presence of antagonist ZM-241385 (10 μM). Values are expressed as the mean ($\pm$SEM) of four (*A* and *C*) or three (*B*) independent experiments. The data shown in *D* are the average of two experiments with similar results.

5*A* and data not shown). As tested in a single experiment, adenosine deaminase treatment (0.25 IU/ml) did not clearly change the basal cAMP levels, suggesting that the impact of endogenous adenosine on the stimulation of cAMP is minimal (data not shown).

Intracellular accumulation of cAMP in TNF-α-treated cells was significantly elevated following stimulation by the highly selective A_{2A} agonist, MRE0094 (1 μM) (21), and reached a peak of 160 $\pm$ 13% of the basal level after 10 min of incubation. In contrast, MRE0094 did not increase cAMP generation in control or IFN-γ-treated cells at any time point (Fig. 5*B*). Moreover, higher doses of MRE0094 only induced a slight increase in the cAMP level in control cells and no response in IFN-γ-treated cells, whereas increasing concentrations of the agonist induced a modest, but significant, dose-dependent increase (up to 165 $\pm$ 14% by 10 μM MRE0094) in the cAMP level in TNF-α-treated cells ($p < 0.01$ vs control for overall response of TNF-α-treated cells to MRE0094; $n = 4$; two-way ANOVA; Fig. 5*C*). ZM-241385 almost completely reversed the effect of MRE0094 on the cellular cAMP concentration, providing further confirmation that the A_{2A} receptor was responsible for the MRE0094-mediated stimulation of increased cellular cAMP concentrations (Fig. 5*D*).

To determine which receptor mediated the generation of cAMP, we further tested cytokine-treated cells with the nonselective adenosine receptor agonist NECA. As with MRE0094, there was little cAMP response to NECA in control cells. Surprisingly, not only TNF-α-treated cells, but also IFN-γ-treated cells, showed a dose-dependent increase in cAMP level after treatment with NECA (66 $\pm$ 15 and 44 $\pm$ 10% increase by 10 μM NECA in TNF-α- and IFN-γ-treated cells, respectively; Fig. 6*A*). The lack of a cAMP response to MRE0094 in IFN-γ-treated cells and the presence of such a response to NECA are consistent with the hypothesis that the A_{2B} receptor can also stimulate cAMP formation in these cells. Moreover, the observation that ZM-241385 does not reverse the effect of NECA on cAMP in IFN-γ-treated cells provides evidence that A_{2B} receptors on HMVEC also stimulate an increment in cAMP (Fig. 6*B*). However, the effect of NECA on TNF-α -treated cells was largely reversed by the A_{2A} receptor antagonist ZM-241385 (Fig. 6B) indicating the prominent role of the A_{2A} receptor in stimulation of cAMP production following cytokine treatment.

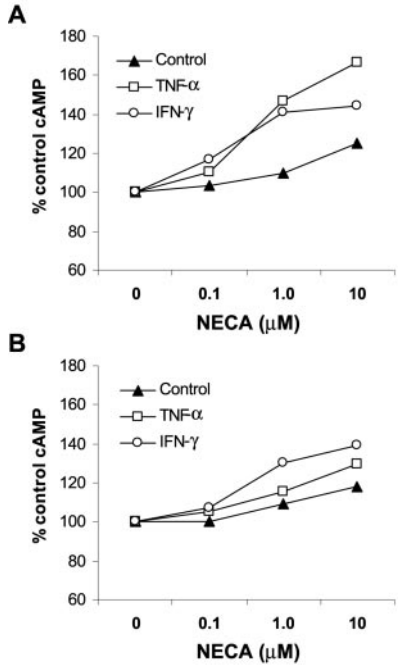

FIGURE 6. Effect of NECA on cAMP accumulation in cytokine-treated HMVEC. *A*, HMVECs with overnight cytokine pretreatment were stimulated for 15 min with the indicated concentrations of NECA. *B*, ZM-241385 (10 μM) was added to the culture before NECA stimulation. Values are expressed as the average of two independent experiments.

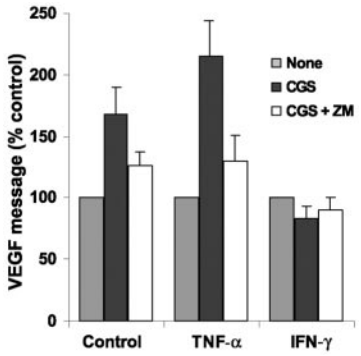

FIGURE 7. TNF-α and IFN-γ differentially modulate the stimulatory effect of the adenosine receptor agonist CGS-2168 on VEGF mRNA expression. HMVECs were pretreated overnight with TNF-α or IFN-γ, then stimulated with CGS-21680 (1 μM) for 4 h in the absence (CGS) or the presence of 1 μM ZM-241385 (CGS + ZM). The expression of VEGF message was quantitated by real-time PCR and is presented as a percentage of the control (none). The basal levels of VEGF in unstimulated cells (without agonist or antagonist) were 1.55 $\pm$ 0.30, 2.02 $\pm$ 0.59, and 1.62 $\pm$ 0.12% of GAPDH mRNA for control, TNF-α-treated, and IFN-γ-treated cells, respectively ($n = 3$).

Effects of TNF-α and IFN-γ on CGS-2168-mediated VEGF mRNA synthesis

To further understand the effect of inflammatory cytokines on the adenosine receptor function, we examined the expression of VEGF mRNA following stimulation with the adenosine receptor agonist CGS-21680 in cytokine-pretreated HMVECs. The level of VEGF mRNA in untreated HMVECs was increased to 168 $\pm$ 21% of the control value by 1 μM CGS-21680 after a 4-h incubation (Fig. 7). More importantly, the stimulatory effect of CGS-21680 on VEGF expression was significantly enhanced in TNF-α-treated cells ($p <$ 0.05 for TNF-α vs control), whereas this effect was completely attenuated in IFN-γ-treated cells ($p < 0.01$ vs control; Fig. 7). The effect of CGS-21680 on VEGF expression in untreated and TNF-α-treated cells was largely, but not completely, reversed by 1 μM ZM-241385 ($p < 0.05$ for both; Fig. 7). The effect of ZM-241385 in control and TNF-α-treated cells and the absence of a VEGF response in IFN-γ-treated cells suggest that increased VEGF synthesis is mainly mediated by the A_{2A} receptor, although the A_{2B} receptor could also be involved. Taken together, the data suggest that the changes in adenosine receptor number produced by the cytokines studied are functionally significant.

IL-1 and TNF-α up-regulate expression of the $G_{\beta 4}$ isoform

The change in A_{2A} receptor expression upon exposure to TNF-α did not correlate well with the more striking functional changes in the receptor with respect to cAMP levels and VEGF production, as shown above and in the previous study of THP-1 cells (19). When activated by adenosine or its analogues, A_{2A} and A_{2B} receptors couple to $G_{\alpha s}$ or G_{olf} protein to stimulate increases in cellular cAMP levels. Recent studies have demonstrated that G protein β isoforms in $\beta\gamma$ dimers determine the strength and specificity of receptor-effector coupling for A_{2A} receptors (24, 25). $G_{\beta 4}$ proteins couple A_{2A} receptors to adenylyl cyclase most efficiently, whereas $G_{\beta 1}$ proteins do not couple A_{2A} receptors to adenylyl cyclase at all (25). To understand the role of inflammatory cytokines on downstream effectors in adenosine receptor signaling, we examined the message and protein expression of $G_{\alpha s}$, $G_{\beta 1}$, and $G_{\beta 4}$ proteins in HMVEC treated overnight with IL-1, TNF-α, or IFN-γ. Surprisingly, the message level of $G_{\alpha s}$ in treated and untreated HMVEC was nearly undetectable by either semiquantitative or quantitative

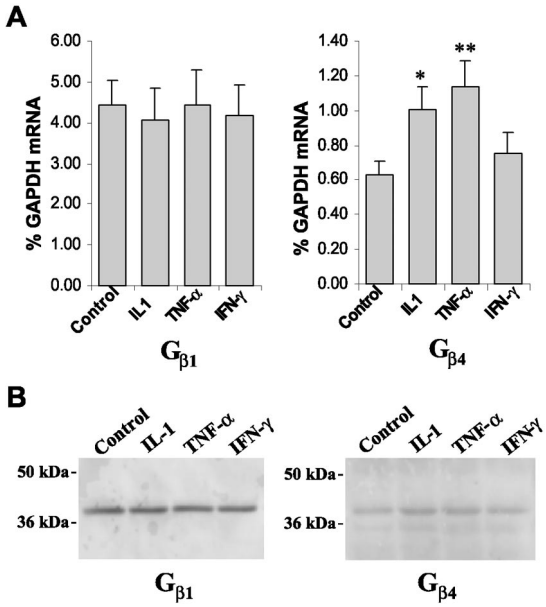

FIGURE 8. Effect of inflammatory cytokines on the expression of $G_{\beta 1}$ and $G_{\beta 4}$ proteins in HMVEC. Cells were treated overnight with IL-1, TNF-α, or IFN-γ, followed by mRNA and protein analyses. *A*, Quantitative real-time PCR was performed to estimate the copy number of cDNA for $G_{\beta 1}$ and $G_{\beta 4}$ proteins, and data are expressed as the percentage of GAPDH cDNA. Data shown are the mean ($\pm$SEM) of four independent experiments. *, $p < 0.05$; **, $p < 0.01$. *B*, Western blot analysis of membrane preparations from treated cells (30–40 μg for each lane) were performed using polyclonal Abs against $G_{\beta 1}$ and $G_{\beta 4}$ proteins. Data shown are representative of three experiments with similar results.

real-time PCR. Immunoblotting of membrane preparations from treated HMVEC failed to detect the appropriate band for $G_{\alpha s}$ protein, although the same Ab could detect such a band for $G_{\alpha s}$ in similar membrane preparations of THP-1 cells (data not shown). Message for G_{olf} was detectable in HMVEC (data not shown), and coupling to this protein probably permits coupling to adenylyl cyclase. Interestingly, the message level for $G_{\beta 1}$ protein in HMVEC, estimated by quantitative real-time PCR, is ~7-fold higher than that for $G_{\beta 4}$ protein (4.45 $\pm$ 0.56% of GAPDH mRNA for $G_{\beta 1}$ vs 0.63 $\pm$ 0.09% for $G_{\beta 4}$ in resting cells; Fig. 8*A*). Overnight treatment with IL-1, TNF-α, or IFN-γ did not affect $G_{\beta 1}$ expression (Fig. 8*A*). In contrast, IL-1 and TNF-α treatment significantly increased the expression of $G_{\beta 4}$ message by ~60 and 80%, respectively, over the control value ($p < 0.05$ for IL-1; $p < 0.01$ for TNF-α). IFN-γ-treated cells showed slightly increased expression of $G_{\beta 4}$ message (Fig. 8*A*). Similarly, Western immunoblotting showed no cytokine-induced changes in protein levels of $G_{\beta 1}$. $G_{\beta 4}$ protein was barely detectable in control cells and increased markedly in the IL-1- and TNF-α-treated cells, although densitometric quantitation of changes in these low levels of expression is not very reliable (Fig. 8*B*).

Discussion

We demonstrate here that cultured human dermal microvascular endothelial cells express both A_{2A} and A_{2B} receptors and, more importantly, that expression of those receptors is differentially regulated at both the message and protein levels by the Th1 inflammatory cytokines IL-1, TNF-α, and IFN-γ. These results parallel the previously reported changes in A_{2A} and A_{2B} receptors in the human monocytoid cell line THP-1 (19). Moreover, the demonstration that IFN-γ up-regulates A_{2B} receptor expression in human

THP-1 cells and endothelial cells is consistent with the effect of this cytokine on murine bone marrow-derived macrophages (18). Up-regulation of A_{2A} receptor by IL-1 and TNF-α in human cells (HMVEC and THP-1 cells) in our studies is also in agreement with a recent report on rat PC12 cells by Trincavelli and colleagues (20). Thus, data from our studies and others suggest that the action of proinflammatory cytokines, such as IL-1, TNF-α, and IFN-γ, on the expression of adenosine receptors does not vary with the cell type studied or the species of origin of the cells, although the consequences of cytokine exposure on the receptor function might be different.

Inflammatory exudates are complex mixtures of inflammatory mediators and regulators; thus, the observation that TNF-α and IL-1 regulate adenosine A_{2A} receptor expression in an opposing manner to IFN-γ may be significant with respect to feedback regulation of inflammation. Our results demonstrate that the effect of TNF-α on A_{2A} receptor expression dominates the effect IFN-γ, suggesting the importance of adenosine acting at A_{2A} receptors as feedback inhibitors of inflammation. Our observations also suggest one mechanism by which desensitization of adenosine A_{2A} receptors at inflamed sites can be overcome, inflammatory cytokines increase receptor expression despite ongoing ligation of the receptors.

It has been reported that adenosine, acting via its receptors, modulates endothelial functions during inflammation. Most notably, adenosine prevents the increase in vascular permeability and thus promotes endothelial barrier function (26–28). At sites of chronic inflammation microvessels proliferate in response to a variety of signals and in previous studies activation of both A_{2A} and A_{2B} receptors on either endothelial cells or macrophages has been reported to enhance the expression of VEGF and promote angiogenesis (4–7), observations consistent with our findings. Although all the factors that regulate adenosine receptor expression and function in vivo have not been explored, the demonstration that adenosine receptors expressed in microvascular endothelial cells are modulated during inflammation suggests an important role for these receptors in the increased angiogenesis and vascular permeability that characterize both acute and chronic inflammatory responses.

In parallel with the analysis of message and protein levels, the pharmacological effects of A_{2A} and A_{2B} receptor agonists on cAMP accumulation provide evidence that the cytokine-induced changes in the expression of these receptors are functionally significant. The results reported here are consistent with the coupling of both A_{2A} and A_{2B} receptors to cAMP accumulation, as previously reported for these receptors (29–31), as demonstrated by the following: 1) the nonselective adenosine receptor agonist NECA stimulates an increase in cellular cAMP content that is partially reversed by the selective A_{2A} antagonist ZM-241385, whereas the A_{2A} selective agonist MRE0094 stimulates an increment in cellular cAMP that is completely blocked by ZM-241385; 2) pretreatment with TNF increases message and protein for both A_{2A} and A_{2B} receptors, whereas IFN-γ diminishes A_{2A}, but increases A_{2B} message and protein, which correlates well with the observation that the A_{2A}-selective agonist MRE0094 stimulates cAMP accumulation in TNF-treated, but not IFN-γ-treated, HMVEC, but the nonselective agonist NECA stimulates cAMP accumulation in cells treated with either cytokine. Interestingly, the magnitude of the change in cAMP observed in HMVEC is much less than that we have previously observed in THP-1 cells (19). Based on the relative level of message and cAMP responses to A_{2A} (CGS-21680) and A_{2B} (NECA) receptor agonists in the human microvascular endothelial cell line HMEC-1, Feoktistov and colleagues (4) have suggested that the dominant adenosine receptor in microvascular endothelial cells is the A_{2B} receptor. Our results suggest that in primary human endothelial cells, HMVEC, both receptors are poorly coupled to adenylyl cyclase under basal conditions, although they are more actively and functionally coupled following cytokine treatment. Moreover, our results suggest that in resting HMVEC disparate levels of message expression for A_{2A} and A_{2B} receptors do not reflect the dominance of one or the other of these receptors at the functional level. The minimal cAMP response of resting HMVEC to the agonists tested here might also be due to low levels of functional A_{2A} and A_{2B} receptor expression and/or a lack of sufficient signal transduction machinery under basal conditions.

Recent studies indicate that the β isoform in the $\beta\gamma$ dimer is important for efficient coupling of activated adenosine receptors to $G_{\alpha s}$ proteins and can serve as a determinant of the specificity of signaling at both receptors and effectors (25). For A_{2A} receptor, $G_{\beta 1}$ does not couple the receptor to adenylyl cyclase, whereas $G_{\beta 4}$ leads to the most effective coupling of A_{2A} receptor to adenylyl cyclase. In fact, $G_{\beta 4}$ subunit provides a high affinity agonist binding to the A_{2A} receptor-G protein complex that contains the subunit (24). Interestingly, we demonstrated that inflammatory cytokines, such as IL-1 and TNF-α, not only up-regulate A_{2A} and A_{2B} receptors, but also enhance the expression of $G_{\beta 4}$ protein. The effect was observed at both message and protein levels, suggesting a direct action of these cytokines on the $G_{\beta 4}$ gene, although the exact mechanism of regulation must be further clarified. Nonetheless, it is suggested that in addition to modulation of adenosine receptor number on the cell surface, these cytokines may also stimulate the expression and/or recruitment of selective G_{β} proteins or other elements in the cell membrane, promoting receptor-G protein coupling and cellular signaling, leading to greatly enhanced changes in receptor-induced function.

Prior work has demonstrated that adenosine and its receptors regulate the secretion of VEGF, a growth factor involved in the regulation of endothelial functions in inflammation and angiogenesis. While some investigators reported a primary role for the A_{2A} receptor in modulation of VEGF secretion (7), others have suggested that the A_{2B} receptor is the primary regulator of VEGF secretion (4, 5). The adenosine A_{2A} receptor agonist CGS21680 stimulated a marked increase in VEGF mRNA expression in HMVECs at a pharmacologically selective concentration (1 μM), which was reversed by an A_{2A} receptor-selective concentration of ZM-241385, consistent with a primary role for A_{2A} receptors in the regulation of VEGF. Moreover, the observation that pretreatment with TNF-α permits significantly greater enhancement of VEGF mRNA by the adenosine receptor agonist, whereas pretreatment with IFN-γ abrogates the CGS21680-mediated stimulation of VEGF mRNA further confirms the role of adenosine A_{2A} receptors in stimulating VEGF mRNA accumulation in HMVECs. However, these data do not rule out the potential role for A_{2B} receptors in the regulation of VEGF mRNA accumulation in these cells.

It is now generally appreciated that there is significant variation in the function of endothelial cells lining various types and sizes of vessels. Nonetheless, we were surprised to observe that, in contrast to HUVEC, which express all four types of adenosine receptor (9), microvascular endothelial cells express only A_{2A} and A_{2B} receptors. In general, A_1 and A_3 receptors, when occupied, antagonize the effects of A_{2A} and A_{2B} receptors as a result of their tight coupling to G_i-linked signal transduction pathways (which down-regulate G_s-mediated signal transduction) (29–32). We speculate that it is possible that the absence of adenosine A_1 and A_3 receptors on microvascular endothelium reflects the role of the microvascular endothelium, as opposed to the macrovascular or arterial endothelium, in angiogenesis and the stimulation of angiogenesis by adenosine acting at both A_{2A} and A_{2B} receptors.

Elevated intracellular levels of cAMP are generally associated with the suppression of inflammatory responses (33, 34). Moreover, it has been firmly established that the increased adenosine secretion and activation of its receptors inhibit the production of many proinflammatory cytokines, including IL-12, TNF-α, and other inflammatory mediators (19, 35–38). It is likely then that at inflamed sites, where proinflammatory cytokines such as IL-1 and TNF-α are abundantly secreted, mostly by monocytes/macrophages, the subsequent up-regulation of A_{2A} and A_{2B} receptors on endothelial cells and other inflammatory cells along with endogenous adenosine release constitutes a feedback loop to suppress further inflammation. Furthermore, we and others have demonstrated that adenosine, acting at its receptors, mediates the anti-inflammatory effect of low dose methotrexate, a widespread and effective therapy for rheumatoid arthritis and other inflammatory diseases (39). IL-1, TNF-α, and IFN-γ are the most important Th1 cytokines involved in the pathogenesis and development of these disorders (for review, see Refs. 40 and 41). We speculate that the actions of these cytokines on modulation of adenosine receptor expression and function may influence the anti-inflammatory effect of methotrexate, and our findings may provide partial explanation for the variation in clinical response to methotrexate therapy.

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