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Research Article

TNF-α upregulates adenosine 2b (A2b) receptor expression and signaling in intestinal epithelial cells: a basis for A2bR overexpression in colitis

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Abstract. Adenosine is an endogenous signaling molecule upregulated during inflammatory conditions. Acting through the A2b receptor (A2bR), the predominant adenosine receptor in human colonic epithelia, adenosine has been directly implicated in immune and inflammatory responses in the intestine. Little is known about expression and regulation of A2bR during inflammation. Tumor necrosis factor alpha (TNF-α) is highly upregulated during chronic and acute inflammatory diseases. This study examined the expression of A2bR during colitis and studied effects of TNF-α on A2bR expression, signaling and function. Results demonstrated that A2bR expression increases during active colitis. TNF-α pretreatment of intestinal epithelial cells increased A2bR messenger RNA and protein expression. TNF-α significantly increased adenosine-induced membrane recruitment of A2bR and cyclic adenosine monophosphate downstream signaling. Further, TNF-α potentiated adenosine-induced short-circuit current and fibronectin secretion. In conclusion, we demonstrated that TNF-α is an important regulator of A2bR, and during inflammation, upregulation of TNF-α may potentiate adenosine-mediated responses.

Key words. T84 cells; TNF alpha; chloride secretion; cAMP; adenosine.

Introduction

Adenosine is a potent endogenous regulator that modulates the function and differentiation of epithelial, inflammatory and immune cells. Its levels increase in the intestinal lumen and in tissue during inflammation to as high as 500–600 nM [1]. The A2b receptor (A2bR), one of the four adenosine receptor subtypes (A1, A2a, A2b and A3), mediates the biological effects of adenosine in the intestine [2, 3]. A2bR is the predominant adenosine receptor expressed in the cecum and colon in the intact human colonic mucosa [3, 4] and the only adenosine receptor expressed in the colonic cell line T84 [3]. In the intestine, apical or basolateral stimulation of A2bR by adenosine results in cyclic adenosine monophosphate (cAMP)-dependent electrogenic chloride secretion through the activation of chloride channels [3, 5]. Active chloride secretion by intestinal crypt enterocytes is known to be the central pathophysiological disturbance in acute and chronic diarrheal illnesses [6]. In addition to chloride secretion, adenosine also induces apically directed interleukin 6 (IL-6) secretion and fibronectin secretion [7, 8]. Adenosine-induced IL-6 secretion has been shown to activate neutrophil calcium response and epithelial intercel-

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lular adhesion molecule 1 (ICAM-1) expression, while adenosine-induced fibronectin secretion has been shown to potentiate the adherence and invasion of *Salmonella typhimurium.* [7–10]. Thus adenosine, acting through A2bR, modulates intestinal secretion, immune and inflammatory response.

In intestinal epithelial cells, A2bR positively couples to G alpha s protein isoform (Gαs) and activates adenylate cyclase. Apical or basolateral stimulation of A2bR induces an increase in intracellular cAMP and downstream cAMP signaling, including phosphorylation and activation of the cyclic AMP response element binding protein transcription factor (CREB), and the activation of protein kinase A (PKA) [3, 11]. The phosphorylated CREB is involved in IL-6 secretion in the intestine in response to adenosine, and PKA is involved in the chloride secretory pathway activated by adenosine [7, 12]. Unlike mast cells, where A2bR also couples to G q protein isoform (Gq) and increases intracellular calcium, cAMP is the only signaling pathway mediated by A2bR in the intestinal epithelial cells [3, 13]. Studies in our laboratory have demonstrated that A2bR undergoes SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) mediated recruitment to the membrane upon agonist stimulation. At the membrane, A2bR exists as a multi-protein complex with PDZ domain-containing protein (PSD postsynaptic density protein, the Drosophila septate junction protein Discs-large and the epithelial tight junction protein ZO-1), NHERF-2 (Na^+/H^+ exchanger regulatory factor 2), cytoskeletal anchoring protein, ezrin and PKA [14, 15].

Although the biological effects of adenosine in the intestine have been characterized, the expression and regulation of A2bR have not been fully elucidated. Several lines of evidence have suggested that the expression and functions of adenosine receptors may be regulated by endogenous factors involved in inflammation, cellular growth and differentiation, such as glucocorticoids, growth factors and cytokines [16–20]. Recent studies in our lab have demonstrated that interferon gamma causes acute downstream regulation of A2bR possibly through inhibition of activity and expression of adenylate cyclase [21]. To further understand the regulation of A2bR during inflammation, we studied the effect of a key proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) on the expression, signaling and function of A2bR. TNF-α is a 17-kDa multi-factorial cytokine produced by many cell types, including macrophages, mast cells, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges**.** The helper T cell (Th1) cytokine elicits a particularly broad spectrum of organismal and cellular responses, including lymphocyte and leukocyte activation and migration, fever, acute-phase response, cell proliferation, differentiation, and apoptosis [22]. TNF-α is highly upregulated during chronic inflammatory diseases such as inflammatory bowel disease (IBD) and during acute viral or bacterial enteritis. TNF-α is thought to play a central role in the pathogenesis of inflammation and diarrhea associated with these diseases [23]. Although a clear mechanism has not been identified to date, anti-TNF-α therapy is successful in reducing symptoms and relapses of IBD [24, 25].

In this study we investigated the expression of A2bR during human and experimental colitis and studied the effect of TNF-α exposure on the expression, signaling and secretory function of A2bR in T84 cells.

Materials and methods

Reagents

Adenosine was obtained from Research Biochemicals Int. (Natick, MA) and was used (at 100 μM) via apical or basolateral stimulation. Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) and nitrocellulose membranes (0.45-μm pores) were from Bio-Rad (Hercules, CA). Anti-A2bR antibody (1:1000 dilution) and control peptide (A2BR 23-P) were from Alpha Diagnostics Inc. (San Antonio, TX), anti-fibronectin (1:3000 dilution) from Sigma (St. Louis, MO), TNF-α from R&D systems Inc. (Minneapolis, MN); forskolin (FSK) was from Calbiochem (San Diego, CA), β-actin antibody (1:200 dilution) was from Santa Cruz biotech (Santa Cruz, CA) and Na^+K^+ATPase (used at 0.1μg/ml) from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA) and isobutylmethylxanthine (IBMX) from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). The cAMP-Screen Kit was from Applied Biosystems (Bedford, MA). The Quantigene Reagent system was from Genospectra (Fremont, CA). ATL-GW64S was a generous gift from Dr. Joel Linden (Adenosine Therapeutics, University of Virginia, Charlottesville, VA)

Cell culture

T84 cells were grown and maintained in culture as previously described [26] in a 1:1 mixture of Dulbecco's modified Eagle's and F-12 medium supplemented with penicillin (40 mg/l), streptomycin (90 mg/l) and 5% newborn calf serum. Confluent stock monolayers were subcultured by trypsinization. Experiments were performed on cells plated for 7–8 days on permeable supports of 0.33 cm^2, 4.5-cm^2 inserts or 1-cm^2 snap well filters (0.4 μm pore size) (Costar, Cambridge, MA). Inserts rested in wells containing media until steady-state resistance was achieved, as previously described. This permitted the apical and basolateral membranes to be separately interfaced with apical and basolateral buffer, a configuration identical to that previously developed for various microassays.

The T84 cells had a high electrical resistance (900–1200 /cm^2, n = 50 monolayers). All experiments were performed on T84 cells between passages 69 and 76.

Confocal microscopy

A2bR expression was evaluated in patients with Crohn's disease (CD) (n = 4) or ulcerative colitis (UC) (n = 4). Infectious colitis was ruled out by stool cultures. The collection of samples was approved by the Institutional Review Board of Emory University. Mucosal biopsy specimens were obtained during routine endoscopy that was performed after written informed consent was obtained (n = 6). Biopsy samples were taken from involved areas of the ileum or colon in CD patients and from actively inflamed colonic segments in UC patients with mild-moderate disease. The diagnosis and extent of CD or UC were established by defined clinical, laboratory, endoscopic/radiological and histopathological criteria. Control biopsy samples were collected from volunteers undergoing colonoscopy for colorectal cancer screening (n = 4). Control biopsy samples were all negative for colorectal cancer. Confocal microscopy was performed in tissue section frozen in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek). Tissue sections were fixed in 3.7% buffered formaldehyde for 20 min, incubated with A2bR antibody (1:100 dilution) overnight in a 4 °C humidity chamber, washed with Hanks Balanced Salt Solution and incubated with fluorsceinated secondary antibodies (Jackson ImmunoResearch). The isotype control was from serum of nonimmunized rabbits in which the primary antibody was raised. Tissues were also counterstained with rhodamine/phalloidin to visualize actin and mounted in *p*-phenylenediamine/glycerol (1:1), and were analyzed by confocal microscopy (Zeiss dual laser confocal microscope) as described [15]. Quantitation of confocal images was performed on unprocessed images using Metamorph Imaging System Software (Universal Imaging Corp., West Chester, PA). The average grayscale pixel intensity +1 standard deviation of a small region was measured and defined as background. To subtract background, the threshold of each channel was set at the value obtained for background. The average pixel intensity +1 standard deviation was measured for the thresholded images [15]. The data are presented as the mean ± S.E.

DSS-induced colitis in mice

Five-week-old C57 black6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were separated into control (water, n = 3) and dextran sodium sulfate (DSS)-treated groups (n = 3). Colitis was induced in mice by the addition of DSS (3% weight-to-volume ratio dissolved in distilled water; mol. wt. 40 kDa) to their drinking water for 5 days after which the mice were sacrificed. The mean DSS water consumption and daily weights were recorded. Stool samples were collected on a daily basis to determine occult blood loss. Severity of colitis was assessed by using a standard protocol which includes (0–12) clinical score based on body weight, diarrhea and occult blood loss [27]. Colonic inflammation and damage were assessed by (0–11) histological scoring which include immune cell infiltration, crypt damage and ulcerations [28].

Processing of mouse tissue for Western blot

Colon was flushed with phosphate-buffered saline (PBS) and everted with the help of a glass rod. The colonic mucosa was scraped gently into PBS on ice, homogenized and centrifuged at high speed. The resulting pellet was reconstituted in Western blot lysis buffer. Equal amounts of protein (50 μg) were separated on SDS-PAGE and transferred onto nitrocellulose paper. The proteins were probed with A2bR antibody (1:1000 dilution) overnight and then incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:4000 dilution) for 1 h. Band detection was performed by chemiluminescence. The blots were also probed in the presence of control blocking peptide (A2B R23-P) to ensure the specificity of mouse A2bR recognized by the antibody.

Preparation of plasma membrane

Plasma membrane fraction was prepared from T84 cells plated in 4.5-cm^2 inserts as described [21]. Briefly, monolayers were washed in PBS, scraped with a rubber policeman and homogenized with a glass/Teflon homogenizer in ice-cold TE buffer (10mM Tris pH 7.5, 50 mM EDTA). The cell suspension was centrifuged at 700 × *g* for 10 min at 4 °C. The supernatant was centrifuged at 17,000 × *g* for 45 min at 4 °C [29]. The pellet enriched in plasma membrane was recovered in lysis buffer containing protease inhibitors. Protein quantitation was done using Lowry method (Biorad, Hercules, CA).

SDS-PAGE and Western blot

Cells were lysed with PBS containing 1% Triton X-100 and 1% Nonidet P-40 (v/v), protease inhibitor mixture (Roche Applied Science), EDTA, SDS, sodium orthovanadate and sodium fluoride. Separation on SDS-PAGE was performed according to the Laemmli procedure. Proteins were electrotransferred onto nitrocellulose membranes and probed with primary antibody (A2bR, Na^+K^+ATPase or β-actin). Membranes were then incubated with corresponding peroxidase-linked secondary antibody, washed and incubated with Western Lightning reagents (PerkinElmer Life Sciences Inc., Boston, MA) according to the manufacturer's directives, before exposure to high-performance chemiluminescence films (Denville Scientific Inc, Metuchen, NJ). For molecular mass (M_r) determination, polyacrylamide gels were calibrated using standard proteins (Bio-Rad) with M_r markers within the range of 10–250 kDa.

Quantitation of Western blot

Western blot band intensity was quantitated using a gel documentation system (Alpha Innotech Co., San Leandro, CA) and represented as arbitary units (AU) in relation to the loading control.

Genospectra QuantiGene mRNA quantitation

A2bR messenger RNA (mRNA) was detected using branched DNA (bDNA) signal amplification analysis. T84 cells were treated with or without TNF-α (10 ng/ml) for various time intervals (1, 2, 4, 6, 12 h). The bDNA signal amplification method has advantages over more commonly used methods for quantifying mRNA in that it uses multiple short oligonucleotides as components of a larger probe set that retains the specificity to discriminate between closely related members of the same gene family without sacrificing sensitivity [30]. Northern blots use large sections of complementary DNA (cDNA) as probes, whereas the bDNA assay uses short oligonucleotides as probes. Additionally, Northern blots rely upon quantification of an autoradiograph, whereas the bDNA assay directly measures luminescence, which is expressed as a numeric value that correlates to the amount of mRNA present. In contrast to reverse transcription-polymerase chain reaction, the bDNA assay works through noncycling, linear amplification, thus eliminating the chance for exponential amplification of a nonspecific-binding event. A2bR mRNA was measured using the bDNA assay (QuantiGene bDNA signal amplification kit, Genospectra). The human A2bR gene sequence was acquired from GenBank and submitted to Genospectra. Multiple oligonucleotide probe sets [capture extender (CE), label extender (LE) and blocker (BL) probes] were designed using Probe Designer software by Genospectra to be specific to a single mRNA transcript. The probes were designed with a T_m of ~63 °C, enabling hybridization conditions to be held constant (i.e. 53 °C) during each hybridization step and for each probe set. All probes designed in Probe Designer were submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known sequences and expressed sequence tags.

Total cell lysate (90 µl) and diluted probe set (10 µl, CE, LE and BL probes for A2bR) were added to the wells of a 96-well capture plate. Total RNA was allowed to hybridize to the probe set overnight at 53 °C. The mRNA transcript of interest is captured by hybridization with CE probes that have also hybridized with oligonucleotides fixed to the bottom of the wells. Subsequent hybridization steps resulted in hybridization of LE probes to the mRNA transcript of interest and to bDNA molecules. The branches of the bDNA molecules hybridized with alkaline phosphatase-conjugated oligonucleotides. Addition of an alkaline phosphatase substrate resulted in generation of luminescence. Luminescence was measured with a Thermolab Systems luminoscan for analysis of luminescence from the 96-well capture plate. The luminescence was reported as relative light units (RLUs), normalized to housekeeping gene 18S RNA.

Northern blot

Northern blot was performed as previously described [31]. Briefly, total RNA was extracted from cells with Tri-reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Total RNA (15 µg) was separated on 1% formaldehyde agarose gel and transferred to Gene Screen Plus membrane (NEN Life Science Products, Boston, MA). After fixation under calibrated ultraviolet (UV) light, the membranes were hybridized with α ^{32}P-labeled A2bR cDNA and visualized by autoradiography. The probe for A2bR was generated using pEGFP-A2b plasmid and digested with Nhe I and Kpn I. 18S RNA was used as a loading control.

cAMP measurement

T84 cells were treated with or without basolateral TNF-α at different concentrations (1, 10, 25, 50, 100 ng/ml) and at different time points (6, 12, 24 h) using10 ng/ml. The cells were stimulated with adenosine (100 µM) and forskolin (10 µM) for 5 min. Total cell lysates were processed in the presence of IBMX (phosphodiesterase inhibitor). cAMP measurements were done in whole cell lysates using a competitive cAMP immunoassay kit (Applied Biosystems, Bedford, MA) according to the manufacturer's directions. Luminescence was read with a Luminoscan Ascent plate reader (Thermo Labsystems, Needham Heights, MA).

Ussing chamber electrophysiology studies

Electrophysiologic studies were performed as described previously [21]. Briefly, T84 cells were plated on snap well filters (12 mm diameter, 0.4 µm pore size, Costar, Cambridge, MA) with a surface area of 1 cm^2 and were grown to confluency (~8 days) until steady-state resistance was achieved. The filter rings were detached and mounted in an Ussing chamber and were incubated with Hanks balanced salt solution at 37 °C and continuously bubbled with 95% O_2, 5% CO_2. The fluid volume on each side of the filter was 5 ml. Voltage-sensing electrodes consisting of Ag/AgCl pellets and current-passing electrodes of silver wire were connected by agar bridges containing 3 M KCl and interfaced via head-stage amplifiers to a microcomputer-controlled voltage/current clamp VCC-MC6, respectively (Physiologic Instruments, San Diego, CA). Voltage-sensing electrodes were matched to within 1 mV asymmetry and corrected by offset-removal circuit. The current between the two compartments (values reported are referenced to the apical side) were monitored and recorded at 20-s intervals, whereas the

voltage was clamped to zero. The voltage was measured with blank filters, first in buffer to be used for the experiments. The values obtained, generally less than 1 mV in magnitude, represent the difference in junction potentials between the two voltage-sensing bridges summed with any potential that might exist across the filter membrane. These values were subtracted from all subsequent measurements using filters containing attached cell monolayers. The total resistance between apical and basal compartments was determined throughout the experiment from the current evoked by a 5-μA bipolar voltage pulse. Before Ussing chamber experiments the cell monolayers were pretreated basolaterally with or without TNF-α (10 ng/ml) at various time intervals from 4 to 24 h. After a sustained baseline short-circuit current (Isc) cells were stimulated with adenosine (100 μM) and forskolin (FSK 10μM). The increase in Isc was then determined.

Measurement of fibronectin secretion

T84 cells plated in 0.33 cm^2 inserts were serum starved with 0.5% FBS for 12 h and pretreated with TNF-α (10 ng/ml for 12 h). Adenosine (100 μM) was then added for 24 h [8]. Media was collected from the apical surface and protein quantification was performed using the Bradford assay. Proteins were electrotransferred to nitrocellulose membranes and probed with fibronectin antibody (1: 1000 dilution). Membranes were incubated with corresponding peroxidase-linked secondary antibody (1: 10,000 dilution), washed and incubated with Western Lightning reagents (PerkinElmer Life Sciences Inc., Boston, MA) according to the manufacturer's directions before exposure to high-performance chemiluminescence films (Denville Scientific Inc, Metuchen, NJ).

Statistical analysis

Statistical analysis was performed using Graphpad Instat 3 software (www.graphpad.com). The severity of colitis in mice is expressed as median with inter-quartile range. All other data are presented as mean ± S.E. The significance of the differences between groups was assessed using the Mann-Whitney *U* test or the Wilcoxon signed-ranked test for nonparametric data and the Student *t* test for parametric data (Isc). p values < 0.05 were considered statistically significant.

Results

A2bR is upregulated in human and DSS-induced murine colitis

To investigate the effect of inflammation and colitis on A2bR expression in intestinal epithelia in vivo, confocal microscopy was performed on intestinal epithelial mucosa from colon biopsies of adult humans with active colitis. Consistent with our previous observation in T84 cells [14, 15], A2bR was predominantly expressed on the basolateral surface and intracellularly in epithelial cells of a normal colonic biopsy (fig. 1A-II). In contrast, the crypt architecture was distorted in IBD tissue, leading to a loss of domain-specific distribution of A2bR (fig. 1A-III). However, A2bR was upregulated in colitis compared with normal subjects. Quantitation of pixel intensity showed a 50% upregulation of A2bR in the colonic mucosa of patients with IBD compared with control subjects. Further, we verified the expression of A2bR during colitis using an established model of murine colitis induced by DSS. All mice given DSS developed bloody diarrhea and weight loss consistent with severe colitis. The histological examination of the colonic mucosa confirmed severe colitis in these mice. Clinical scores were: control 1 (normal range = 0–2), DSS 11 (colitis range = 7–12). Histological scores were: control 1.2 (normal range = 0–2.2), DSS 8.4 (colitis range = 7–10.9), median with inter-quartile range, $p < 0.001$. Western blot performed on the colonic mucosa obtained from mice treated with DSS showed upregulation of A2bR with a 3.2-fold increase compared with control mice given water (fig. 1B).

TNF-α upregulates A2bR mRNA and protein expression

In order to determine the regulation of A2bR during inflammation, we studied the effect of TNF-α, a potent proinflammatory Th1 cytokine that is implicated in the pathogenesis of intestinal inflammation and whose antagonism is an effective treatment of IBD. T84 cells were pretreated for various time points with TNF-α. Northern and Western blots were performed on cell lysates as described in the 'Methods' section. Pretreatment of T84 cells with TNF-α showed an increase in mRNA expression evident at 2 h, maximal at 4 h and return to baseline by 6 h (fig. 2A). In addition to this method we have quantitated A2bR mRNA directly with the QuantiGene system as described in the 'Methods' section. As shown in figure 2B, A2bR mRNA levels were higher at 2 and 4 h compared with control and returned to baseline at 12 h. This correlates with the increase in total A2bR protein in whole cell lysate starting at 4 h. β-Actin was used as loading control (fig. 2C).

TNF-α potentiates A2bR plasma membrane recruitment

Earlier studies in our laboratory demonstrated that A2bR is recruited to the membrane upon agonist stimulation, and membrane recruitment is essential for its signaling [14, 15]. To determine the effect of TNF-α on A2bR membrane recruitment, T84 cells were pretreated basolaterally with TNF-α for 12 h and stimulated with adenosine for 5 min. Plasma membrane extraction and Western blot were performed as described in the 'Methods' section. As shown in figure 3A, TNF-α alone did not increase A2bR membrane recruitment. However, in the presence of adenosine, TNF-α significantly increased

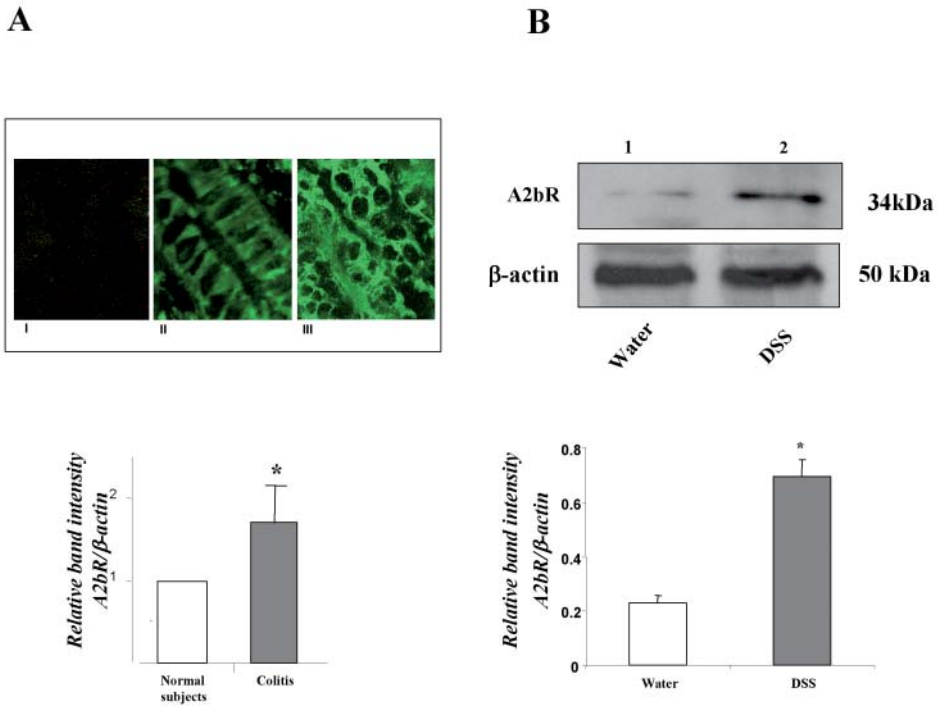

Figure 1. A2bR is upregulated during active colitis in humans and mice. (*A*) Confocal image of A2bR from biopsies of patients with CD. Tissue sections were fixed and stained with human polyclonal A2bR antibody (1:100 dilution) or isotype control antibody (I) followed by fluorescein-ated secondary antibodies (green). The en face images document the upregulation of A2bRs on the membrane in active colitis (III) compared with normal colon (II). The bottom panel shows the pixel intensity of A2bR relative to β-actin obtained as described in 'Methods' section; mean ± S.E., n = 4. (*B*) Western blot analysis of colonic mucosa was performed in control (n = 3) and DSS (3%)-treated (n = 3) C57 Black 6 mice. Equal amounts (50 μg) of proteins were loaded on 4–20% SDS-PAGE and probed with A2bR antibody. Lane 1 represents the control group and lane 2 the DSS-induced colitis group. The bar chart shows the band intensity of total A2bR to β-actin (AU) from the colonic mucosa; mean ± S.E., n = 3, *p < 0.006, significantly higher compared with control group. Representative blots from three independent experiments are shown.

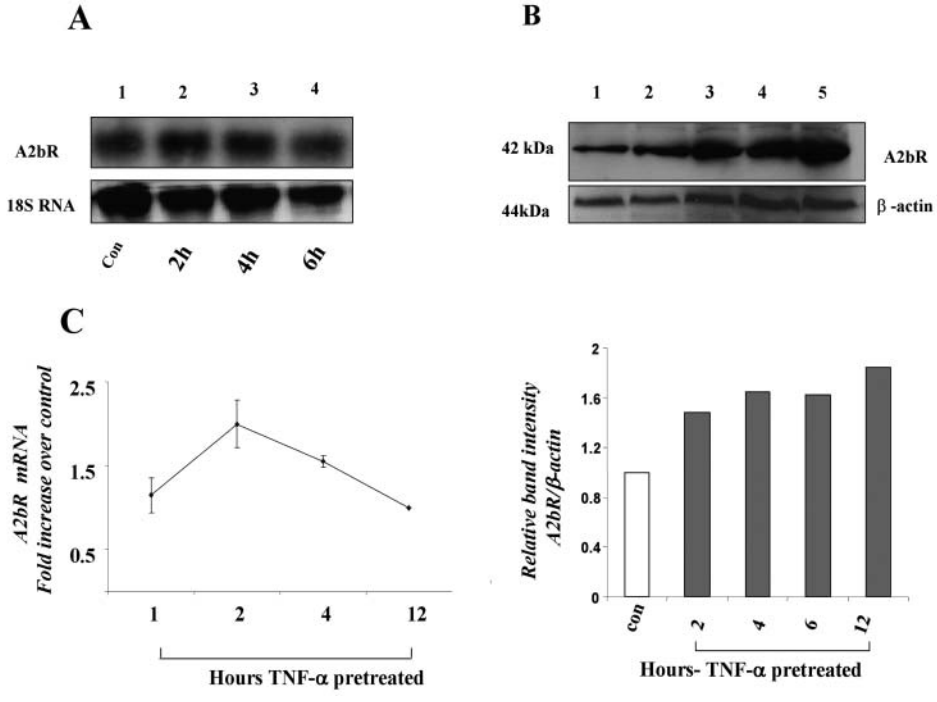

Figure 2. TNF-α upregulates A2bR mRNA and protein. (A) Total RNA was extracted from T84 cells after treatment with TNF-α (10 ng/ml) at 2, 4, 6 h. Northern blot was performed as described in the 'Methods' section using ^{32}P A2bR probes. Lane 1 represents 0 h, lanes 2–4 the respective time points. mRNA expression levels of A2bR were quantitated using 18S RNA for normalization. (B) Total cell lysates were extracted after pretreatment of T84 cells with TNF-α 10 ng/ml at 1, 2, 4, 12 h. QuantiGene analysis was performed as described in the 'Methods' section using an A2bR probe. The Y-axis represents fold increase of A2bR over control. The X-axis represents pretreatment of cells at various time intervals. mRNA levels were normalized with 18S RNA. (C) Western blot analysis of A2bR expression in whole cell lysate of T84 cells after pretreatement with TNF-α (10 ng/ml) at 2, 4, 6, 12 h. Equal amounts (25 μg) of protein were separated by 4–20% SDS-PAGE and probed with human A2bR antibody. Lane 1 represents control, lanes 2–5 the respective time points. β-Actin serves as a loading control; n = 3. Representative blots from three independent experiments are shown.

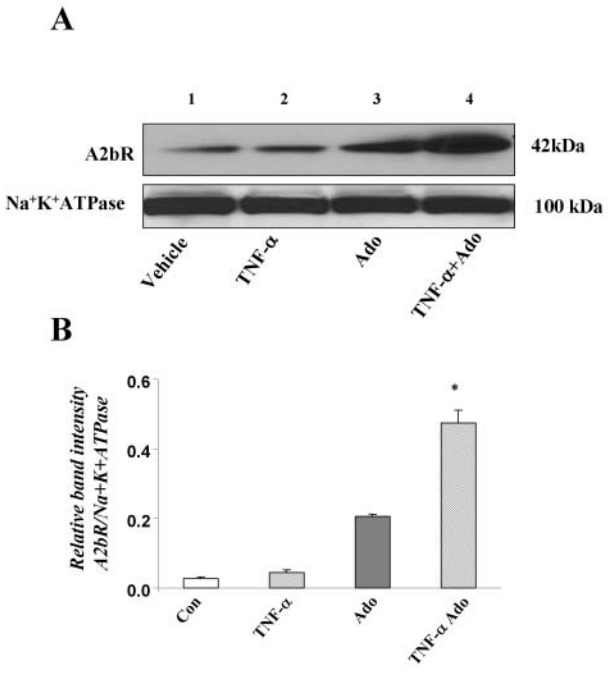

Figure 3. TNF-α potentiates A2bR membrane recruitment. T84 monolayers were pretreated with or without TNF-α 10 ng/ml for 12 h and stimulated with adenosine (100 μM) for 5 min. (A) Equal amounts (25 μg) of plasma membrane fractions were subjected to Western blotting with A2bR. Lane 1 represents control, lane 2 TNF-α treatment alone, lane 3 adenosine stimulation alone and lane 4 TNF-α pretreatment with adenosine stimulation. Na^+K^+ATPase serves as a loading control. (B) represents densitometric quantification of A2bR membrane recruitment under each condition; mean ± S.E., n = 9, *p < 0.001, significantly different from control groups. Representative blots from three independent experiments are shown.

A2bR membrane recruitment (2.25-fold increase when compared with adenosine alone, and 12.5-fold increase compared with control). The levels of Na^+K^+ATPase used as a membrane marker did not change with TNF-α treatment. The bar chart shows the relative band intensity of A2bR membrane recruitment (fig. 3B). The recruitment of A2bR to the membrane was abrogated in the presence of a proteosome inhibitor MG132, which is given as ratio of relative band intensity (A2bR/β-actin), (untreated = 0.59, Ado = 0.72, TNF-α + Ado = 0.81, TNF-α + Ado + MG132 = 0.60).

TNF-α potentiates adenosine-mediated cAMP

A2bR positively couples to Gαs and activates adenylate cyclase. It was previously shown that apical or basolateral stimulation of A2bR induces an increase in intracellular cAMP [3]. Therefore, we studied the effect of TNF-α on adenosine-induced cAMP. T84 cells were pretreated with or without TNF-α for 12 h, and adenosine-stimulated cAMP levels were quantitated using a luminometric assay as described in the 'Methods' section. As expected, adenosine increased cAMP levels, which were maximal at 5 min after stimulation (~5-fold increase compared with control). As shown in the table 1**,** pretreatment of cells with TNF-α resulted in an increase in adenosine-stimulated cAMP levels by ~11-fold compared with control and 2-fold compared with adenosine stimulation alone. Interestingly, TNF-α had no effect on forskolin-mediated cAMP increases. Further, A2bR-specific antagonist, ATL GW64S (500 nM) [14], inhibited adenosine-induced cAMP in TNF-α pretreated cells by 94–96%. These results demonstrated that TNF-α specifically potentiates adenosine-induced cAMP levels, suggesting receptor-mediated upregulation of adenosine's effect.

Table 1. TNF-α potentiates adenosine-induced cAMP levels

Treatment	cAMP fold increase over control
TNF-α	1.3 ± 0.4
Adenosine	5.1 ± 1.9
TNF-α	11.6 ± 2.1*
TNF-α + Adenosine + ATLGW64S	1.3 ± 0.8
Forskolin	5.3 ± 1.7
TNF-α + Forskolin	5.2 ± 1.5

T84 monolayers were pretreated with TNF α (10 ng/ml) for 12 h and then stimulated with adenosine (100 μM) or forskolin 10 μM for 5 min. The cellular cAMP levels were measured as described in the 'Methods' section. Fold increases in cAMP levels compared with control cells (absence of TNF-α and Ado) are shown in column 2. cAMP levels in TNF-α pretreated cells were significantly higher than the levels in cells stimulated with adenosine alone. It is also shown that ATLGW 64S (A2bR antagonist) completely abolished increases in cAMP levels by adenosine. Data represent the mean ± S.E., n = 6, *p < 0.01.

TNF-α potentiates adenosine-induced cAMP in a time- and dose-dependent manner

Figure 4A shows the dose-dependent response of TNF-α. We observed that TNF-α potentiated cAMP increases up to 10 ng/ml (~12-fold increase compared with control, and a 1.7-fold increase compared with adenosine alone) and 25 ng/ml (~15-fold increase compared with control, and 1.8-fold increase compared with adenosine alone). At higher concentrations of TNF-α (50 and 100 ng/ml for 12 h), we observed an inhibiton of adenosine-mediated cAMP levels, probably due to the apoptotic effects of the cytokine. TNF-α potentiated adenosine-induced cAMP levels after 6-h pretreatment with TNF-α (~12-fold increase compared with control, and 2-fold increase compared with adenosine stimulation alone). This was further increased (~23-fold increase compared with control and ~10-fold increase compared with adenosine stimulation) after pretreatment for 12 or 24 h (fig. 4B).

TNF-α potentiates adenosine-induced chloride secretion and fibronectin secretion

We previously showed that adenosine mediates chloride (Isc) and fibronectin secretion. Since TNF-α potentiated

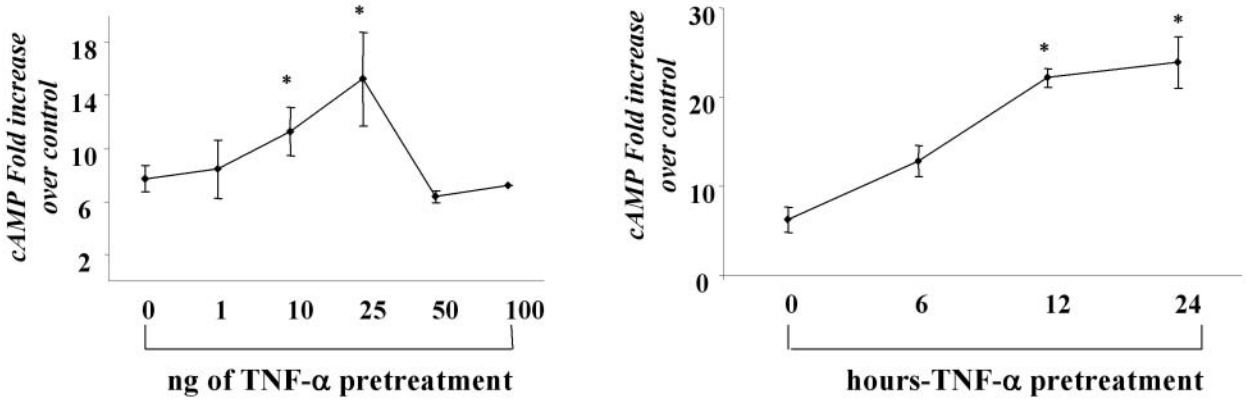

Figure 4. TNF-α potentiates adenosine-induced cAMP in a dose- and time-dependent manner. (A) T84 cells were pretreated with TNF-α 1,10, 25, 50 and 100 ng/ml for 12 h and then stimulated with adenosine (100 μM) (Ado) for 5 min. The cellular cAMP levels were measured as described in the 'Methods' section. Fold increases in cAMP levels compared with control cells were detremined from three independent experiments; mean ± S.E., n = 6. (B) cells were pretreated with TNF-α (10ng/ml) for 6, 12 and 24 h and stimulated with adenosine (100 μM) for 5 min. Fold increases in cAMP levels compared with control cells were determined from three independent experiments, each using two wells per condition; mean ± S.E., n = 6.

adenosine-mediated signaling, we determined the effect of TNF-α pretreatment on adenosine-induced chloride and fibronectin secretion. T84 monolayers were grown on snap wells. Snap wells were then mounted on Ussing chambers, stimulated with adenosine, and Isc was measured. Cells were pretreated with TNF-α for 12 h. Monolayers were stimulated with adenosine after a sustained baseline current. TNF-α alone for 12 h had no effect on basal Isc or transepithelial resistance measured. TNF-α treatment significantly increased adenosine-induced Isc (TNFα + Ado = 65.8 ± 6.43μA/cm^2) compared with adenosine alone (Ado = 35.8 ± 5.93 μA/cm^2) (fig. 5A, B). To determine whether TNF-α also potentiates adenosine-mediated fibronectin secretion, confluent monolayers of intestinal epithelial T84 cells, grown in tissue culture inserts, were stimulated with adenosine, and conditioned media was analyzed by Western blot analysis to examine the level of the protein. Fibronectin levels in the T84 culture media were significantly increased in adenosine-treated cells, and TNF-α pretreatment further potentiated the fibronectin secretion (fig. 5C). These results demonstrated that TNF-α potentiates A2bR-mediated functions, including chloride and fibronectin secretion in intestinal epithelial cells.

Discussion

Using colon tissue from humans and DSS-treated mice, which have been found to have cytokine levels similar to those in human IBD [32], we demonstrated for the first time that A2bR is upregulated in colitis. Given that A2bR is the predominant adenosine receptor in colonic epithelial cells, our findings are of pathophysiological significance, as adenosine levels are increased during inflammation and adenosine mediates immune and inflammatory pathways in the intestine [1, 3, 33]. Several studies have addressed the regulation of adenosine receptors in immune (monocytes, macrophages, lymphocytes) and endothelial cells during inflammatory conditions [16]. A2bR has been shown to be upregulated by Th1 cytokines, TNF-α and interleukin (IL)-1 in endothelial cells [34]. Our interest in TNF-α stems from the fact that it is a pivotal cytokine with diverse proinflammatory effects within the intestinal mucosa. Evidence for the role of TNF-α in human IBD has accumulated over the years, demonstrating unequivocally that TNF-α is a critical mediator of inflammatory and immune responses in colitis. TNF-α levels are significantly increased in intestinal mucosa, serum and stools in Crohn's disease and to a lesser extent in ulcerative colitis [35–39]. Anti-TNF-α therapy is effective in decreasing intestinal permeability and disease activity in IBD [24, 25]. Interestingly, we observed that TNF-α increases mRNA and expression of A2bR protein. Thus, given the pleiotropic effect of adenosine on intestinal function, upregulation of A2bR by TNF-α constitutes a novel downstream effector pathway for TNF-α.

Previous studies in our laboratory have shown that membrane recruitment is required for signaling of A2bR [15]. Our results show that TNF-α treatment of T84 cells for 12 h potentiated adenosine-induced plasma membrane recruitment of A2bR. We observed that although TNF-α increases total intracellular A2bR, the agonist is crucial for recruitment to the membrane to take place. Our results demonstrated that TNF-α potentiated adenosine-mediated cAMP levels in a time- and dose-dependent manner. Consistent with previous findings, we observed

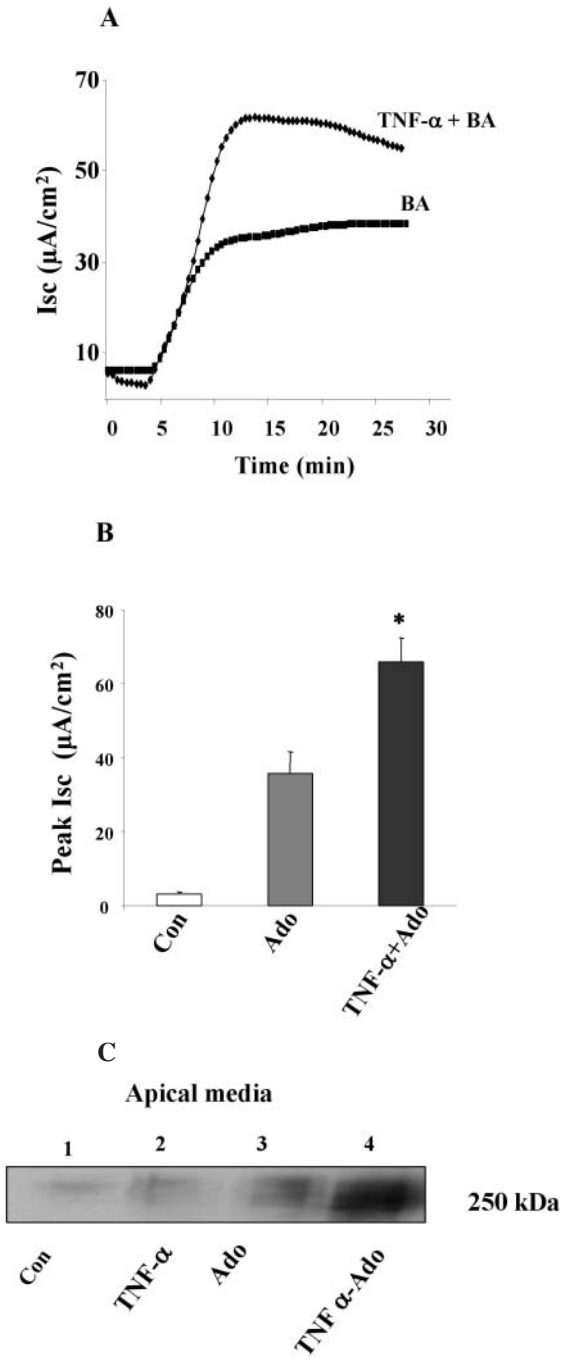

Figure 5. TNF-α potentitates adenosine-mediated Isc and fibronectin secretion. (A and B) T84 cells were grown on snap wells and mounted on Ussing chambers after pretreatment with TNF-α 10ng/ml for 12 h. After a sustained baseline Isc, cells were stimulated with adenosine (100 μM) (Ado). The increase in Isc was determined as described in the 'Materials' section. On the left is a representative tracing from the Ussing chamber and the right shows peak Isc, mean ± S.E, n = 3, *p < 0.002 significantly different from adenosine stimulation alone. (C) Confluent monolayers were serum starved, pretreated with TNF-α (10ng/ml) for 12 h in serum free media and then stimulated with (100 μM) (Ado) for 24 h. The conditioned media was subjected to Western blot using fibronectin antibody. Lane 1, control; lane 2, TNF-α treatment alone; lane 3, adenosine alone; lane 4, TNF-α + Ado, which shows increased band intensity. Representative blots from four independent experiments are shown (n = 4).

that TNF-α pretreatment alone does not increase cAMP levels [34]. Moreover, it does not potentiate an increase in cAMP by forskolin, a direct adenylate cyclase activator. Further, TNF-α-mediated increase in cAMP is inhibited by an A2bR antagonist. Taken together, these data confirm that TNF-α exerts a specific effect on A2bR signaling. However, the concomitant presence of adenosine, as seen during inflammation, is necessary to mediate recruitment and downstream signaling of the receptor. These data also imply that during an inflammatory state where TNF-α is abundantly secreted (mostly by monocytes and macrophages), the subsequent upregulation of A2bR along with the endogenous release of adenosine constitutes a previously unexplored pathway of inflammatory response.

The mechanism by which TNF-α increases A2bR levels is not known. It is possible that TNF-κ/NF- B (nuclear factor kappa B) may directly activate A2bR expression by activating A2bR promoter. In this context, we observed that MG-132, a proteosome inhibitor, abrogates TNF-α-induced A2bR expression, suggesting NF-κB may directly influence A2bR synthesis. A secondary mechanism of action of TNF-α may involve enhanced production of adenosine in vivo, as has been suggested with prostaglandin E2 and histamine [40, 41]. Such increase in adenosine may potentiate membrane recruitment of A2bR, thus increasing receptor expression on the membrane. Further studies are needed to elucidate the mechanism by which TNF-α induces A2bR expression.

Regulation of ion secretion and barrier function by TNF-α has been extensively studied. It has been demonstrated that TNF-α does not directly stimulate intestinal ion transport. Rather, prolonged exposure to highly elevated concentrations of TNF-α may reduce barrier capacity to macromolecules. [42–44]. Our results support observations of others showing the absence of a response with TNF-α treatment alone or its pretreatment on forskolin-mediated Isc [45]. In the HT29cl 19A colonic epithelial cell line, TNF-α pretreatment potentiated carbachol- and histamine-induced chloride secretion via upregulation of the protein lipase D pathway, although it had no effect on the muscarinic or histamine receptors, respectively [40]. In this study, we observed that TNF-α potentiated adenosine-induced ion secretion by upregulation of the receptor itself.

In summary, we have demonstrated that A2bR is upregulated during human and murine colitis. TNF-α induced the expression of A2bR and potentiated adenosine-induced signaling and functional responses in intestinal epithelial cells. The demonstration that A2bR, expressed by intestinal epithelial cells, is modulated during inflammation suggests an important role for these receptors in the regulation of secretory responses, neutrophil function and bacterial epithelial interaction that characterize acute and chronic inflammation.

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