Adenosine \( A_{2A} \) and \( A_{2B} \) Receptors Differentially Modulate Keratinocyte Proliferation: Possible Deregulation in Psoriatic Epidermis

Rosa M. Andrés\(^1\), María Carmen Terencio\(^1\), Jorge Arasa\(^1\), Miguel Payá\(^1\), Francisca Valcuende-Cavero\(^1\), Pedro Navalón\(^5\) and María Carmen Montesinos\(^1\)

Adenosine is a potent regulator of inflammation and immunity, but the role of adenosine receptors in keratinocytes remains controversial. We determined that in addition to \( A_{2B} \) receptors, human epidermal keratinocytes also express \( A_{2A} \) receptors, although to a lower extent. Through the use of selective adenosine receptor agonists and antagonists, we showed that physiological concentrations of adenosine activate \( A_{2B} \) receptors in normal human keratinocytes, inducing cell cycle arrest through the increase of intracellular calcium but not through cAMP signaling. In contrast, the selective activation of \( A_{2A} \) receptors by CGS-21680 induces keratinocyte proliferation via p38–mitogen-activated protein kinase activation. Adenosine and selective \( A_{2A} \) and \( A_{2B} \) agonists presented anti-inflammatory profiles independent of adenosine receptors but mediated by membrane phosphatase activation. Finally, keratinocyte exposure to diverse inflammatory cytokines altered adenosine receptor expression by reducing \( A_{2B} \) and increasing \( A_{2A} \), a pattern also observed in psoriatic epidermis. Because increased epidermal turnover and inflammatory response are characteristics of psoriatic disease, further studies are needed to assess the role and consequences of the altered adenosine receptor expression in lesional and nonlesional psoriatic keratinocytes.


INTRODUCTION

In the era of biologic therapies, the classic immunomodulator methotrexate is still considered a first-line, inexpensive systemic treatment of psoriasis with a very well-established safety profile (Yelamos and Puig, 2015). Methotrexate is a competitive inhibitor of dihydrofolate reductase, which blocks DNA synthesis and cell mitosis of rapidly dividing cells. However, folic acid supplements are co-administered to reduce its toxicity and adverse effects without compromising its anti-inflammatory efficacy, suggesting that other mechanisms of action might exist (Bangert and Costner, 2007). Although no single mechanism is sufficient to account for all the anti-inflammatory activities of methotrexate, several studies have shown the involvement of adenosine (Chan and Cronstein, 2010; Yelamos and Puig, 2015).

Adenosine is an endogenous purine nucleoside that can be released or formed by enzymatic dephosphorylation of adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate during inflammation, wounding, and other pathological states. Once in the extracellular compartment, adenosine acts through four subtypes of adenosine receptors (ARs), \( A_1, A_2A, A_2B, \) and \( A_3 \), all belonging to the large family of G protein-associated receptors (Fredholm et al., 2011). Numerous studies indicate that adenosine is a potent regulator of inflammation and immunity (Antonioli et al., 2014; Ernst et al., 2010). In contrast, little is known about adenosine effects on epidermal cells, and reports are often contradictory. Thus, activation of adenosine \( A_{2B} \) receptors promotes murine keratinocyte proliferation (Braun et al., 2006), whereas adenosine inhibits human keratinocyte proliferation through uptake by the cell membrane transporter hENT1 (Brown et al., 2000).

Purinergic signaling in healthy and diseased skin has emerged as a renewed area of interest (Burnstock et al., 2012). In particular, activation of adenosine \( A_{2A} \) receptor plays an important role in promotion of wound healing (Montesinos et al., 2015), being especially relevant in the formation of new granulation tissue and revascularization of the wound bed (Montesinos and Valls, 2010; Valls et al., 2009). Although mice with a targeted disruption of this receptor subtype showed a defect in granulation tissue formation, wound re-epithelialization was hardly compromised.
Intracellular cAMP and iCa²⁺ in NHEK with the selective A2A agonist CGS-21680 (CGS), the response of adenosine (Ernst et al., 2010). We incubated has been mainly associated with the anti-inflammatory and, thus, their activation increases intracellular cAMP, which has been also characterized as an A2A AR antagonist (Flutter and Nestle, 2013; Schon et al., 2006). Furthermore, an A1 AR agonist is currently being evaluated in phase II and III clinical trials in patients with moderate to severe plaque psoriasis because of its regulatory effect on T-cell function (David et al., 2012; Kofoed et al., 2015).

In this study we have assessed the expression and signal transduction mechanisms of AR in normal human epidermal keratinocytes (NHEK). In addition, through the use of selective AR agonists and antagonists, we have shown a differential effect of A2B and A2A AR on cell proliferation and a receptor-independent anti-inflammatory effect. Finally, we have determined the possible influence of the inflammatory milieu on AR expression in NHEK, which mimics the expression of AR in psoriatic epidermis.

RESULTS

Adenosine A2B but not A2A receptor stimulation increases intracellular cAMP and iCa²⁺ in NHEK

Earlier reports indicated that A2B was the only AR subtype expressed by NHEK (Brown et al., 2000), whereas, in murine keratinocytes, lower expressions of A2A, and A3 AR have also been described (Braun et al., 2006). We analyzed the expression of AR in NHEK by reverse transcriptase PCR and confirmed that, although A2B is the major AR subtype expressed by these cells (ΔCt against glyceraldehyde-3-phosphate dehydrogenase = 7.02 ± 0.074, n = 10), A2A receptors are also expressed to a lesser extent (ΔCt against glyceraldehyde-3-phosphate dehydrogenase = 12.50 ± 0.16, n = 12, P < 0.001 vs. A2B), whereas subtypes A1 and A3 were undetectable (Figure 1a).

Both A2A and A2B ARs are supposedly coupled to Gs proteins and, thus, their activation increases intracellular cAMP, which has been mainly associated with the anti-inflammatory response of adenosine (Ernst et al., 2010). We incubated NHEK with the selective A2A agonist CGS-21680 (CGS), the selective A2B agonist BAY60–6583 (BAY), or the nonselective agonist 5’-N-ethylcarboxamidoadenosine (NECA) alone or in combination with the selective antagonists SCH-442416 (SCH) and MRS-1706 (MRS) (see Supplementary Table S1 online). After 15 minutes, NECA and BAY induced a significant increase in the intracellular cAMP concentration that was inhibited by the selective A2A antagonist MRS, indicating signaling through Gs protein (Figure 1b). In contrast, CGS failed to induce cAMP elevation, and the selective A2A antagonist SCH did not affect NECA-induced production of cAMP, ruling out this second messenger downstream of the A2A AR activation in NHEK.

Because A2B AR has also been reported to signal through intracellular calcium (iCa²⁺) via Gq protein activation in certain cellular types (Aherne et al., 2011; Haskó et al., 2008), we incubated NHEK with AR agonists and antagonists and determined iCa²⁺ levels by coupling with a fluorescent dye. As shown in Figure 1c, stimulation with either the nonselective agonist NECA or the selective A2B agonist BAY led to an increase in iCa²⁺ that was reversed by co-incubation with the A2B antagonist MRS but not by the A2A selective antagonist SCH; the A2A agonist CGS had no effect at this level. These results are consistent with A2B AR signaling through Gq in NHEK as well.

Adenosine A2A and A2B receptor subtypes differentially modulate keratinocyte proliferation

Using selective AR agonists and antagonists, we found that activation of A2A and A2B AR had opposing effects on NHEK proliferation, determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Thus, incubation with the selective A2B agonist BAY decreased keratinocyte proliferation, whereas the selective A2B agonist CGS increased cell growth (Figure 2a), effects that were inhibited by the selective antagonists MRS and SCH, respectively.

The nonselective agonist NECA, widely used in research as an adenosine equivalent that does not suffer enzymatic deamination, enhanced keratinocyte proliferation. This effect was abrogated by the selective antagonist SCH, indicating the involvement of A2A AR. Because it has been shown that adenosine inhibited proliferation of NHEK (Brown et al., 2000), we determined its effect in the presence or absence of the selective antagonists. As shown in Figure 2a, the anti-proliferative effect of adenosine was primarily mediated by A2B AR, because it was abrogated by MRS. The opposing effects of the nonselective agonist NECA and the endogenous ligand adenosine are probably due to the higher affinity of NECA for the A2A AR (see Supplementary Table S1) (Alnouri et al., 2015), thus explaining the previously reported divergences regarding the effect of AR activation on NHEK proliferation.

Cell proliferation results were confirmed by cell cycle analysis. As shown in Figure 2b, A2A stimulation by CGS significantly increased the fraction of cells in G2/M in accordance with the increased cell proliferation observed by MTT assay, even though there was no appreciable change in the S phase. In contrast, A2B activation decreased S and G2/M fraction while increasing G1, showing an antiproliferative effect. These results were validated with selective AR antagonists (see Supplementary Figure S1 online). Similar to the MTT assay, NECA and adenosine evidenced opposing effects in the cell cycle mediated by A2A and A2B receptors, respectively (Figure 2b, and see Supplementary Figure S1).

Opposite effects of A2A and A2B AR on the cell cycle are mediated by p38 mitogen-activated protein kinase and iCa²⁺, respectively

Because A2B AR signaled through cAMP in NHEK, we evaluated the effect of the adenylyl cyclase activator forskolin on keratinocyte cell growth. In contrast to the effect of adenosine and BAY, forskolin highly increased proliferation (Figure 2a and b), suggesting that other signaling pathways rather than cAMP mediate the cell cycle arrest induced by A2B activation in NHEK. To determine the possible participation of downstream targets to the cAMP signaling cascade, cells were incubated with the protein kinase A inhibitor H-89 (10 μmol/L) and the exchange protein activated by cAMP (EPAC) ESI-09 (10 μmol/L). Unfortunately, both inhibitors showed toxicity, determined by lactate dehydrogenase and trypan blue exclusion (data not shown) to warrant reliable results.
As stated before, AR activate additional signaling pathways apart from G\textsubscript{s} proteins, such as extracellular signal-regulated kinases and p38 mitogen-activated protein kinase or G\textsubscript{q/-mediated increase of iCa\textsuperscript{2+}} (Aherne et al., 2011). Given the pivotal role of these signaling pathways in the control of the cell cycle, we pre-incubated NHEK with the selective inhibitors PD98059 (extracellular signal-regulated kinase 1/2), SB202190 (p38), or the calcium chelator 1,2-Bis(2-aminomino)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxyethyl ester) (BAPTA-AM) and determined the cell cycle distribution after 24-hour stimulation with AR agonists. All these inhibitors did not affect cell cycle at the concentrations assayed (see Supplementary Figure S2 online). BAPTA-AM significantly reversed the antiproliferative effect of adenosine (Figure 3a) and BAY (Figure 3b), decreasing the proportion of cells in G\textsubscript{1} and suggesting that calcium mediates, at least in part, the partial cell cycle arrest induced by A\textsubscript{2B} activation. On the other hand, the p38 inhibitor SB202190 partially blocked the effect of A\textsubscript{2A} AR activation, reversing the increased fraction of cells in the G\textsubscript{2}/M phase induced by CGS (Figure 3c).

Adenosine and AR agonists inhibit the inflammatory response of NHEK

Epidermal keratinocytes are key participants in innate or adaptive immune responses through production of cytokines and chemokines such as tumor necrosis factor (TNF-\(\alpha\)) or IL-8 (Lowes et al., 2014) and adenosine is a well-established regulator of inflammation (Haskó and Cronstein, 2013). Therefore, we determined the effect of AR activation on cytokine release by NHEK stimulated with the protein kinase C activator TPA, which can reproduce certain inflammatory parameters of psoriatic skin in animal models (Andres et al., 2013). Adenosine, as well as the selective A\textsubscript{2A} and A\textsubscript{2B} agonists, inhibited the levels of TNF-\(\alpha\) and IL-8 in a concentration-dependent manner; however, this effect was not reversed by any of the selective antagonists (Figure 4). These results suggested an unspecific anti-inflammatory mechanism independent of ARs. This controversial point has been previously reported in other cell types such as neutrophils and macrophages, indicating that adenosine can suppress cell activation and TNF-\(\alpha\) release by other independent mechanisms such as the activation of membrane phosphatases (Fotheringham et al., 2004; Haskó and Cronstein, 2004; Kreckler et al., 2009). In this sense, pre-incubation of NHEK with the serine/threonine protein phosphatase inhibitor okadaic acid enhanced TNF-\(\alpha\) release induced by the protein kinase C activator TPA as previously described (Fujiki et al., 2013). Nevertheless, in the presence of okadaic acid, all tested AR agonists failed to inhibit cytokine release after TPA stimulation (see Supplementary Figure S3 online).
Adenosine Receptors on Keratinocyte Function

**DISCUSSION**

In this study we have elucidated the contribution of AR in controlling keratinocyte proliferation, resolving a long-lasting controversy. We have determined that adenosine promotes cell-cycle arrest, in agreement with older reports (Brown et al., 2000; Cook et al., 1995); however, using much lower concentrations and selective antagonists, we have shown the involvement of A2B AR. On the other hand, we have shown that A2A receptor mRNA was increased (Figure 5b). Accordingly, immunoblotting analysis confirmed that psoriatic epidermis showed higher expression of A2A and lower expression of A2B ARs than foreskin epidermis at the protein level, despite a certain degree of interindividual variability (Figure 5c), possibly because of receptor glycosylation, as previously reported (Linden et al., 1999). Therefore, these results suggest that some inflammatory mediators generated in psoriatic skin could deregulate the normal ratio of AR expression in epithelium and consequently alter the physiological role of AR in keratinocytes.

**Inflammatory milieu could alter AR expression in NHEK and psoriatic epidermis**

Several reports have described that the constant exposure of inflammatory cytokines modulate AR expression in both infiltrating and resident cells (Khoa et al., 2001; Morello et al., 2006; Nguyen et al., 2003; Xaus et al., 1999). Consequently, we hypothesized that the inflammatory milieu characteristic of psoriatic plaques could alter AR expression in NHEK. To assess this hypothesis, we determined mRNA levels of A2A and A2B receptors in NHEK preincubated with an array of pro-inflammatory cytokines known to be up-regulated in psoriasis (TNF-α, IL-1β, IL-6, IL-17, IL-23, IFN-α, or IFN-γ) or with the protein kinase C activator TPA. We observed that IFN-γ, and the reference stimulus TPA, altered the expression of both ARs, decreasing A2B and increasing A2A. On the other hand, IFN-α significantly decreased A2B AR expression, whereas TNF-α and IL-1β increased A2A AR. Other psoriatic mediators such as IL-6, IL-17, and IL-23 had no effect (Figure 5a). In view of these results, we next compared the pattern of AR expression in epidermis obtained from biopsy samples of plaque-type psoriasis versus epidermis from surgical foreskin resections of healthy donors, used throughout this study as the source of NHEK. In healthy epidermis, AR expression was similar to the one described earlier for NHEK (Figure 5b). However, even though A2B continued to be more expressed than A2A, and A1 and A3 were undetectable in psoriatic epidermis, we observed a significantly different level of expression of both receptor subtypes compared with normal epidermis: A2B receptor expression was reduced, whereas A2A receptor mRNA was increased (Figure 5b). Accordingly, immunoblotting analysis confirmed that psoriatic epidermis showed higher expression of A2A and lower expression of A2B ARs than foreskin epidermis at the protein level, despite a certain degree of interindividual variability (Figure 5c), possibly because of receptor glycosylation, as previously reported (Linden et al., 1999). Therefore, these results suggest that some inflammatory mediators generated in psoriatic skin could deregulate the normal ratio of AR expression in epithelium and consequently alter the physiological role of AR in keratinocytes.

**Figure 2. Effect of adenosine receptor stimulation on NHEK cell growth.**

(a) Proliferation was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay after 48 hours of treatment. Data are mean absorbance at 490 nm ± standard error of the mean (n = 8–10). *P < 0.05, **P < 0.01, ***P < 0.001 versus basal cells (nonstimulated) using Dunnett test. †P < 0.05, ‡P < 0.01 using Student t test. (b) Results of cell-cycle analysis by flow cytometry after 24 hours of treatment. Data are mean ± standard error of the mean (n = 10–12). *P < 0.05, **P < 0.01, ***P < 0.001 versus the same distribution phase in the basal cell cycle (Dunnett t test). CGS-21680, BAY60–6583, adenosine, and NECA were tested at 1 μmol/L. The antagonists SCH-442416 (A2A selective) and MRS-1706 (A2B selective) were pre-incubated for 30 minutes at 1 μmol/L. The adenylyl cyclase activator forskolin was tested at 50 μmol/L. ADO, adenosine; BAY, BAY60-6583; CGS, CGS-21680; Forsk, forskolin; MRS, MRS-1706; NECA, 5′-N-ethylcarboxamidoadenosine; NHEK, normal human epidermal keratinocyte; ns, not significant; SCH, SCH-442416.
with no involvement of cAMP. Calcium regulates the growth, differentiation, and apoptosis of many cell types, including epidermal keratinocytes, in which it induces $G_1/G_0$ cell cycle arrest (Bikle et al., 2012). Thus, some antipsoriatic drugs such as 1α,25-dihydroxyvitamin D$_3$ or fumaric acid exert an antiproliferative effect mediated by iCa$^{2+}$ elevation (Dascalu et al., 2000). In contrast to the A$_{2B}$-mediated antiproliferative effect of adenosine, activation of A$_{2A}$ AR induces a proliferative response in NHEK dependent on p38 and independent of cAMP. This observation is in agreement with several reports stating that the peculiar long C-terminus of A$_{2A}$ AR is responsible for this behavior in NHEK. Additionally, the lack of intracellular calcium could be defective in skin autoimmune diseases such as psoriasis (Kiehl and Ionescu, 1992; Kose et al., 2001); overexpression of A$_{2A}$ and A$_3$ AR in inflammatory cells has been found in different autoimmune disorders (Ochaion et al., 2009; Varani et al., 2011). In this study, we have observed a reduction in A$_{2B}$ AR expression besides the increased expression of A$_{2A}$ AR in psoriatic epidermis. Furthermore, some inflammatory mediators released during the psoriatic process could be in part responsible for these variations, particularly cytokines such as IL-1β, TNF-α, IFN-γ, and IFN-β, which are mainly involved in the initiation of the psoriatic plaque (Perera et al., 2012). Moreover, such cytokines as IL-1β and TNF-α have been shown to regulate A$_{2A}$ AR function as well by preventing its desensitization (Khoa et al., 2006).

On the other hand, the IL-17/IL-23 axis cytokines, characteristic of the established psoriatic plaque, had no effect. Therefore, it seems reasonable to think that the variations in AR expression could occur during the early stages of lesion development. In this regard, the up-regulation of A$_{2A}$ AR by TNF-α and IL-1β could play a protective anti-inflammatory role, as other researchers have suggested (Borea et al., 2016). Otherwise, IFN-γ produced by plasmacytoid dendritic cells is one of the key and initial steps in psoriasis pathogenesis contributing to keratinocyte proliferation (Farkas and Kemeny, 2012). Thus, the reduction of A$_{2B}$ expression after stimulation with IFN-γ could cause the loss of the antiproliferative control elicited by this receptor subtype in NHEK. However, further experiments comparing

$$A_{2B} \text{ AR mediated the protective effect of adenosine in intestinal inflammation by promoting epithelial barrier (Aherne et al., 2015).}$$

Our results also indicate that intracellular calcium mediates the antiproliferative effect of A$_{2B}$ AR activation on NHEK,
lesional to nonlesional psoriatic skin will need to be conducted to confirm this hypothesis.

This study has shown an intriguing dual role of A$_{2A}$ and A$_{2B}$ ARs on keratinocyte proliferation and their possible deregulation in psoriatic epidermis. The consequences of the altered AR expression on adenosine effects in psoriatic keratinocytes will need to be assessed in further studies, given the high potential of this endogenous mediator to ameliorate inflammatory diseases. Our results suggest that adenosine plays an important role in regulating epidermal inflammation and keratinocyte function and thus may constitute an interesting therapeutic strategy for inflammatory hyperproliferative skin diseases such as psoriasis.

**MATERIALS AND METHODS**

**Statement on use of human materials**

All protocols and procedures were approved by the University of Valencia Ethical Committee and conformed to the Helsinki guidelines. Patient consent for experiments was not required, because Spanish laws consider human tissue left from surgery as discarded material.

**Isolation, culture, and stimulation of primary human keratinocytes**

Primary human keratinocytes were isolated from foreskins of healthy young donors as described previously (Andres et al., 2013). Briefly, skin samples were treated with a dispase solution and trypsinized. Keratinocytes were grown (37°C/5% CO$_2$) in a serum-free low-Ca$^{2+}$ (<0.1 mol/L) Defined Keratinocyte-SFM (Invitrogen, Carlsbad, CA). For all experiments, cells were seeded at passage numbers 1–3 and treated upon reaching 60–80% confluence. The day before the experiments, medium was replaced to growth factor-free keratinocyte medium.

**Adenosine agonists and antagonists**

AR agonists and antagonists (see Supplementary Table S1) were purchased from Tocris (Bristol, UK) and dissolved in DMSO. The concentration of DMSO in all experiments was less than 0.1%. In all the experiments, antagonists were added 30 minutes before agonists.
Intracellular cAMP determination
NHEK seeded in 24-well plates were stimulated with AR agonist for 15 minutes. Cells were harvested, and intracellular cAMP was assessed using Cyclic AMP Direct EIA Kit (Arbor Assays, Ann Arbor, MI).

\( \text{iCa}^{2+} \) determination
\( \text{iCa}^{2+} \) was assessed using Fluo-4 NW Calcium assay kit (Invitrogen, Carlsbad, CA), following the manufacturer’s recommendations. Briefly, NHEK seeded in 96-well plates were incubated for 1 hour with the dye loading solution and were stimulated with AR agonists \((10 \mu \text{mol/L})\). Fluorescence was measured at different time points using a Wallac 1420 VICTOR2 (PerkinElmer, Waltham, MA).

Proliferation assay
NHEK were seeded in a 12-well plate and incubated for 48 hours with AR agonists \((1 \mu \text{mol/L})\) in growth factor-free keratinocyte medium. Cell density was determined by MTT reduction, and cytotoxicity was assessed by measuring lactate dehydrogenase release in the supernatants (Andres et al., 2013).

Cell cycle assay
AR agonists \((1 \mu \text{mol/L})\) were added to NHEK cultured in 25 cm\(^2\)-flasks with growth factor-free keratinocyte medium. After 24 hours, cells were trypsinized, and cell cycle was measured using BD Cycletes Plus DNA Reagent Kit (BD Biosciences, San Jose, CA). Results were analyzed using a BD FACS VERSE cytometer (BD Biosciences).

Determination of cytokine release
NHEK seeded in 24-well plates were incubated with AR agonists for 30 minutes before stimulation with TPA from Sigma-Aldrich (St. Louis, MO). After 7-hour supernatants were collected. TNF-\(\alpha\), IL-23, IL-6 (50 ng/ml), or TPA (1 \(\mu \text{g/ml}\)) for 3 hours. Subsequently, mRNA levels of \(A_2A\) and \(A_2B\) adenosine receptors were evaluated by quantitative real-time reverse transcriptase-PCR. Data are mean ± standard error of the mean \((n = 6–8)\). *\(P < 0.05\), ***\(P < 0.001\) versus nontreated normal human epidermal keratinocytes using Dunnett t test. All values were normalized to the housekeeping gene GAPDH.

\( \text{A}^2\alpha\text{AR mRNA expression} \)

\( \text{A}^2\beta\text{AR mRNA expression} \)

\( \text{A}^2\alpha\text{protein expression} \)

\( \text{A}^2\beta\text{protein expression} \)

\( \text{A}^2\beta\text{AR} \) mRNA expression in psoriatic versus normal dermis. Data are mean ± standard error of the mean \((n = 10–12)\). *\(P < 0.05\), ***\(P < 0.001\) using the Mann-Whitney test. (c) \(A_{2A}\) and \(A_{2B}\) AR immunoblotting of whole tissue homogenates of normal (N1–N3) and psoriatic epidermis (P1–P3). B, nontreated normal human epidermal keratinocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Adenosine Receptors on Keratinocyte Function


Montesinos MC, Desai-Merchant A, Cronstein BN. Promotion of wound healing by an agonist of adenosine A receptor is dependent on tissue plasminogen activator. Inflammation 2015;38:2036–41.


