Novel Kinin B₁ Receptor Splice Variant and 5′ UTR Regulatory Elements Are Responsible for Cell Specific B₁ Receptor Expression

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Abstract

The kinin B₁ receptor (B₁R) is rapidly upregulated after tissue trauma or inflammation and is involved in cancer and inflammatory diseases such as asthma. However, the role of the: promoter; a postulated alternative promoter; and spliced variants in airway epithelial and other lung cells are poorly understood. We identified, in various lung cell lines and leucocytes, a novel, naturally occurring splice variant (SV) of human B₁R gene with a shorter 5′ untranslated region. This novel SV is ~33% less stable than the wild-type (WT) transcript in lung adenocarcinoma cells (H2126), but does not influence translation efficiency. Cell-specific differences in splice variant expression were observed post des[Arg¹⁰]-kallidin stimulation with delayed upregulation of SV compared to WT suggesting potentially different regulatory responses to inflammation. Although an alternative promoter was not identified in our cell-lines, several cell-specific regulatory elements within the postulated alternative promoter region (negative response element (NRE) −1020 to −766 bp in H2126; positive response element (PRE) −766 to −410 bp in 16HBE; −410 to +1 region acts as a PRE in H2126 and NRE in 16HBE cells) were found. These findings reveal complex regulation of B₁R receptor expression in pulmonary cells which may allow future therapeutic manipulation in chronic pulmonary inflammation and cancer.

Introduction

Kinins [bradykinin, des[Arg⁹]-BK, and des[Arg¹⁰]-kallidin (DAKD)] are biologically active peptides formed by the enzymatic action of the classical tissue (KLK1) and plasma (KLKB1) kallikreins on endogenous protein substrates called H- and L-kininogens. Kinins are primarily pro-inflammatory and can affect processes such as cell proliferation and migration [1]. These effects are mediated through two G-protein coupled kinin receptors, one of which is the kinin B₁ receptor (B₁R) [2,3]. Kinin B₁ receptor is usually latent under normal physiological conditions but is quickly upregulated following initiation of inflammatory pathways [4–7].

Airway epithelial cells not only provide a protective lining but also initiate and regulate airway inflammation and tissue repair. The airways are constantly exposed to both exogenous and endogenous stimuli including antigens, particulates, chemicals, mediators and pathogens. Epithelial cell stimulation frequently results in inflammation which following repeated insults may lead to cell death, fibrosis and epigenetic changes that may favour tumorigenesis [8]. It has been suggested that B₁R plays a role in sustaining and amplifying chronic inflammation [9]. It is expressed in pulmonary epithelial cells including human bronchial epithelial cells (16HBE and BEAS-2B) and human lung adenocarcinoma (A549) cell lines [9,10]. Furthermore, B₁R expression is induced in inflammation and tissue injury [11,12]. Thus, tobacco smoke increases expression of B₁R expression in rat trachea [13], leading to increased airway hyperresponsiveness [14]. B₁R is also involved in bronchial hyperresponsiveness in rodent models of asthma [15,16]. In humans, B₁R expression is increased in eosinophils from asthmatic patients [17] and in nasal tissue of patients with allergic rhinitis after allergen challenge which was not observed in healthy subjects [9]. Dexamethasone, however, reduced basal expression of B₁R and suppressed its upregulation by proinflammatory stimuli [18]. These studies all suggest that B₁R expression is involved in the pathogenesis of chronic inflammation in allergic and smoke-related diseases such as asthma, lung cancer and COPD.

Despite its significant importance, the regulation of B₁R expression is not clear. Human B₁R gene contains three exons, with the first and the second being non-coding. Characterisation of the 5′ flanking core promoter region has shown the presence of a functional TATA-box and other regulatory elements that are cell-specific [19]. A positive regulatory element (PRE) functioning as an enhancer has been identified at −604 to −448 bp while a negative regulatory element (NRE) that ablates the enhancer activity is identified at −602 bp to −604 bp region relative to the
transcription start site (TSS) [19,20]. Detailed footprint analysis of the promoter region suggests possible binding by several transcription factor sites such as GATA-1, PEA3, AP-1, CAAT, Sp1, Pit-1a, Oct-1 and CREB [21].

It has been suggested there is a second, alternative promoter, located in intron II, as well as additional regulatory elements [20,22,23]. This region demonstrates cell specific activity [18]. While this region shows stronger basal promoter activity than the core promoter in HepG2 cells, it exhibits properties of a weaker promoter in vascular smooth muscle cells [18]. Whether this region functions as a promoter, particularly in inflammation as an inducible promoter, is still debated. So far, a single TSS in the kinin B1 receptor has been identified supporting the existence of only a single core promoter [21,23].

In the current study we investigated the existence and function of this putative B1R alternative promoter in human pulmonary cells. While no additional TSS was found a novel 5'UTR splice variant was identified and the expression and function of the novel B1R SV and wild-type WT along with the role of 5'UTR regulatory elements was investigated further in a variety of lung cells. Our findings reveal that a novel B1R splice variant and promoter regulatory elements determine tissue-specific B1R expression.

Methods

Culturing human airway immortalised cell-lines

16HBE, A549, NHLF, HFLF, H520 and H2126 (Table 1) cells were obtained from the American Type Culture Collection (Rockville, MD). 16HBE, NHLF and HFLF were cultured in complete growth media comprised of Dulbecco's modified Eagle's medium (DMEM; Invitrogen), while A549, H520 and H2126 cells were cultured in RPMI 1640 (Invitrogen). All cell-lines were supplemented with 10% fetal bovine serum (GIBCO Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in a humidified atmosphere in 5% CO2 at 37°C. Initial cell seeding density was 20,000 cells/cm2. When cell confluence reached 80%, cells were subcultured by incubating with 0.05% trypsin-0.5 mM ethylenediaminetetraacetate (Invitrogen) at a ratio of 1:3–1:4, weekly.

For cell stimulation purposes, cells were incubated in serum-free media (Invitrogen) as serum has been shown to stimulate B1R expression. Normal cell culture media was replaced with serum-free media for 12 hr prior to the start of stimulation, the cells were then washed once with 1X PBS before being incubated in the absence and presence of the B1R agonist des-Arg9-KD (DAKD)/LPS (Sigma Aldrich) at 100 nM and 1 µM or lipopolysaccharide (LPS)/Sigma Aldrich) at 0.1 µg/µl, for 3, 6 and 24 hr.

Table 1. Lung cell lines screened for B1R mRNA expression.

| Cell lines | Description |
|------------|-------------|
| HFLF       | Human fetal lung fibroblasts |
| NHLF       | Adult human lung fibroblasts |
| H2126      | Human lung adenocarcinoma |
| A549       | Human lung adenocarcinoma |
| 16HBE      | SV-40 transformed normal human bronchial epithelial |
| H520       | Human lung squamous cell carcinoma |

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PCR conditions

PCR amplification reactions were carried out in a reaction mix containing 1X PCR buffer, 1.5 mM – 2.5 mM MgCl2, 5 µM of each dATP, dGTP, dCTP and dTTP (Promega, Madison, WI), 10 pmoles of each forward and reverse primer (Invitrogen or GeneWorks) and 1 U of Taq polymerase (Qiagen). For each PCR reaction, 30 ng-100 ng of DNA was used as a template and the reaction was made up to 25 µl with PCR grade water (Fisher Biotech). PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min; 35 to 40 cycles of product amplification at 94°C for 30, 58–65°C for 30 s, 72°C for 30–60 s; final extension at 72°C for 5 min and finally, temperature hold at 4°C.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 16HBE and H2126 cells using RNeasy mini kits (QIAGEN) as described by the manufacturer and quality confirmed with sharp 28S and 18S ribosomal bands on denaturing agarose gel electrophoresis with ethidium bromide staining. Single-stranded cDNA was generated using Omniscript reverse transcriptase (QIAGEN) in a 20-µl reaction mixture containing reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KC1, 3 mM MgCl2, 10 mM dithiothreitol), 0.5 mM dNTP, 0.5 µg oligo(dT)(Invitrogen), 10 U rRNasin (Promega, Madison, WI), and 2 µg of total RNA. The reaction was incubated for 1 h at 37°C.

Amplification of cDNA by PCR was performed using oligonucleotide primer pairs (GeneWorks, Australia) for the human B1 receptor and the internal control superoxide dismutase 1 (SOD1; Table 2). SOD1 was used as an internal control as its expression was consistent under different stimulation conditions as verified prior to commencing real-time PCR. The reactions were performed in a 25-µl reaction mixture containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KC1, 2.5 mM MgCl2, 0.2 mM dNTP, 2.5 U Taq polymerase (QIAGEN), and 1–2 µl of cDNA. Each primer was added at a final concentration of 0.2 µM. PCR was for 30 to 35 cycles, each cycle consisting of 30 s denaturation at 94°C, annealing at 60°C for 20 s, and extension at 72°C for 50 s. PCR reaction products were separated on 2% agarose gels containing 50 µg/ml ethidium bromide and visualized under UV light.

Real-time PCR

Reactions for real-time PCR contained 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 100 nM of each primer. The PCR conditions were 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were generated after amplification. Data were collected using the iQ5 real-time PCR machine (BioRad). Each sample was tested in duplicate. The standard curve with serial dilutions of cDNA of known concentration was used in each qPCR assay to accurately determine the expression of splice variants, while SOD1 was used for normalisation.

Identification of the B1R transcription start site/s -5’

Rapid Amplification of cDNA Ends (5’-RACE) PCR was performed on 5 µg of total RNA isolated from H2126 cells using the GeneRacer™ kit (Invitrogen). Briefly, cDNA prepared using the GeneRacer™ kit (Invitrogen) was amplified using a B1R gene specific reverse primer RT Rev 3, and GR 5’ Primer, a forward primer that anneals to ligated GeneRacer™RNA oligo. Nested PCR was performed using GR5’ nested and RTRev2 primers (conditions as above). PCR products were subsequently analysed.
Table 2. Primers used in this study.

| Primer   | Description                                                                 | Sequence (5' → 3')                      |
|----------|-----------------------------------------------------------------------------|-----------------------------------------|
| B1R Fow2A| Forward primer for B1R detection in RT-PCR                                  | CCCAACATACAGTTGGAAGCG                   |
| B1R Rev 1| Reverse primer for B1R detection in RT-PCR                                  | CCAGGTTAGATTCTGCCGACG                   |
| B1R Rev  | Reverse primer for B1R detection in RT-PCR                                  | GGGGGGAGATGTAGCTGAAT                    |
| B1R WT F | Forward primer for B1R WT detection in RT-qPCR                              | TTGCTGGACACAGGACTTACT                   |
| B1R SV F | Forward primer for B1R SV detection in RT-qPCR                              | CATTTCGGTGACTGACT                      |
| B1R Rev qPCR | Reverse primer for B1R WT and SV detection in RT-qPCR                  | GCTTCTGGAGCTAGTCAACAG                  |
| Sod1 Fow | Forward primer for SOD1 detection in RT-PCR                                 | GAGAGACATGTTGGAAGACTTG                  |
| Sod1 Rev | Reverse primer for SOD1 detection in RT-PCR                                 | TTCACTGGACCACAGCTGTC                   |
| BglII Fow| Forward primer for E2I2-Luc cloning                                         | ACAGATCTGTCAGTTGCGCTGAGGAGACT           |
| HindIII Rev| Reverse primer for E2I2-Luc cloning                                         | GCTAAGCTTCCTCAAGAACTAGACAGAAGG          |
| B1R Fow  | Forward primer for B1R detection in RT-PCR                                  | GCCCTTTTCAGGTCACAGTGC                   |
| Mlu Fow B1R core | Forward primer for CP-Luc and CP-E2I2-Luc cloning                  | TCCGAAATGTCAGCTACCTA                    |
| Nhel Rev B1R core | Forward primer for CP-Luc and CP-E2I2-Luc cloning                  | GCTAGCTGAGGCAAGAATGAGGGCCGT             |
| B1R HindIII -21R | Reverse primer for E2I2 Δ410-Luc, E2I2 Δ766-Luc, CP-E2I2 Δ410-Luc and CP-E2I2 Δ766-Luc cloning | ACTGAAGCTTGACAGTGACCTGAAATGGAC        |
| B1R Xhol -766F | Forward primer for E2I2 Δ766-Luc and CP-E2I2 Δ766-Luc cloning             | AATCTCGAGACACCCGGCCATACCTCATGTT        |
| B1R Xhol -410F | Forward primer for E2I2 Δ410-Luc and CP-E2I2 Δ410-Luc cloning             | AATCTCGAGCGCTGTAGATCCTGGACAACAGCC       |
| GR 5′ Primer | Primers supplied from Invitrogen for 5′ RACE                           | GGACACTGACATGACTAGGAAGAGTGA            |
| GR 5′ Nested | PCR that binds to the RNA oligo                                    | ACTGACATGACAGTGAAGAGAGTA                |
| RT Rev 3 | Reverse primer for 5′ RACE PCR                                              | TTAGTACCGCGTTGTCAGAC                   |
| RT Rev 2 | Reverse nested primer for 5′ RACE PCR                                      | CAGATATCTCTGGCAGAAGGG                  |

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Construction and transformation of wild-type and splice variant B1R 5′UTR

pGL3 Control vector (Promega) was digested with HindIII and NarI restriction enzymes and the vector backbone was gel extracted using the QIAquick Gel extraction kit (Qiagen, Valencia, CA). B1R WT and SV 5′UTR were amplified using primers that contained HindIII and NarI restriction sites in its 5′-ends (Table 2). The PCR cycling conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 65°C for 20 s, and 72°C for 50 s. The final extension step was at 72°C for 5 min. The PCR products were purified and digested with the HindIII and NarI restriction enzymes and ligated with the pGL3 Control backbone with T4 DNA ligase at 4°C overnight. Transformation into the Escherichia coli strain JM109 was performed by heat shock. Purification of plasmid was performed with QiAprep spin miniprep kit (Qiagen). Constructs were sequenced in the forward and reverse direction to confirm product fidelity.

Sequence analysis

Sequences were aligned and analysed using the program Clustal W (http://align.genome.jp/).

In silico sequence analysis of putative transcription factor binding sites in the B1R alternative promoter (1020 bp upstream of B1R exon III) was performed using Transcription Factor Search (Copyright 1994-2000, Yutaka Akiyama).

Transient Transfections and Luciferase Assays

Transfections of 16HBE and H2126 were performed in 96-well tissue culture plates at a 70% confluence using Lipofectamine 2000 (Invitrogen). pRL-TK (Promega, Madison, WI) was used as a control for measuring transfection efficiency. The transfection
mRNA stability

Actinomycin D (ActD) was used as a highly specific inhibitor of the formation of new RNA. In this experiment, 5 μg/ml Act D was added to 16HBE and H2126 cells subcultured in 6-well plates 24 h after seeding (90% confluence). The cells were harvested at 0, 1, 3 and 5 h after treatment with Act D, and total RNA extracted using RNasea mini kits (Qiagen). The quantity of transcripts was determined using transcript-specific real-time PCR primers (Table 1). The use of a maximum time of 5 h was based on previous studies where total B1R mRNA half-life was documented to be well under 5 h [7,24].

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by a Tukey’s post-test or by Student’s t-test (GraphPad Prism 5.0). P<0.05 were considered to be statistically significant.

Results

Human lung cell lines express varying levels of basal kinin B1 receptor mRNA

Since the degree of B1R expression in airway epithelial and other lung cells is unknown, the basal mRNA expression in six lung cell lines was assessed (Table 1). All cell-lines constitutively expressed B1R receptor except for the lung squamous carcinoma cell line H520 (Fig 1). In the 16HBE cell line, expression of B1R was very low and we were able to detect it only on using real-time RT PCR. Expression of total B1R was the highest in the human fetal lung fibroblast (HFLF) cell line.

Kinin B1 receptor promoter activity in human lung cell lines

We assessed the role of two reported B1R promoters and especially the role of cell specific activity in exon II [22] and intron II [22,23] in regulating B1R promoter activity in the high expressing (H2126) and low expressing (16HBE) lung cell-lines. In the high expressing human lung adenocarcinoma H2126 cell-line, the complete promoter with exon 2 and intron 2 construct (CP-E2I2-Luc, p = 0.003) and the promoter alone construct (CP-Luc, p<0.001) showed significantly increased expression compared to pGL3 Basic (data not shown). While luciferase expression for the promoter alone construct (CP-Luc) was 60% higher (p = 0.004), the exon 2-intron 2 deletion constructs (~1020 bp to ~766 bp; CP-E2I2Δ766-Luc) did not significantly alter luciferase expression in 16HBE (Fig. 2C), compared to the complete construct (CP-E2I2-Luc). Additional removal of ~766 bp to ~410 bp (CP-E2I2 Δ410-Luc) significantly decreased (p = 0.018) luciferase expression compared to CP-E2I2Δ766-Luc. Stimulation of H2126 and 16HBE cells with 100 nM or 1000 nM DAKD for 3, 6 and 24 h did not significantly change the expression level of the promoter constructs (CP-E2I2-Luc, CP-Luc) compared with unstimulated cells (data not shown). Exposure to the general inflammatory stimulus lipopolysaccharide (LPS) 0.1 μg/μL for 3, 6 and 24 h also showed no significant change in luciferase activity (data not shown).

Multiple transcription start sites of B1R and identification of 5’UTR splice variant

In the low expressing human bronchial epithelial cell-line (16HBE), constructs with the complete promoter with exon 2-intron 2 construct (CP-E2I2-Luc, p = 0.003) and the promoter alone construct (CP-Luc, p<0.001) showed significantly increased expression compared to pGL3 Basic (data not shown). While luciferase expression for the promoter alone construct (CP-Luc) was 60% higher (p = 0.004), the exon 2-intron 2 deletion constructs (~1020 bp to ~766 bp; CP-E2I2Δ766-Luc) did not significantly alter luciferase expression in 16HBE (Fig. 2C), compared to the complete construct (CP-E2I2-Luc). Additional removal of ~766 bp to ~410 bp (CP-E2I2 Δ410-Luc) significantly decreased (p = 0.018) luciferase expression compared to CP-E2I2Δ766-Luc. Stimulation of H2126 and 16HBE cells with 100 nM or 1000 nM DAKD for 3, 6 and 24 h did not significantly change the expression level of the promoter constructs (CP-E2I2-Luc, CP-Luc) compared with unstimulated cells (data not shown). Exposure to the general inflammatory stimulus lipopolysaccharide (LPS) 0.1 μg/μL for 3, 6 and 24 h also showed no significant change in luciferase activity (data not shown).
Regulation of Kinin Bₑ Receptor Expression

A

Kinin B₁ receptor gene structure

- 5' promoter
- Exon I
- Exon II
- Exon III
- Introns I and II

-1020bp
-766bp
-410bp

Active promoter in 16HBE and H2126
NRE in H2126
PRE in H2126
PRE in 16HBE and NRE in 16HBE

B

Diagram showing luciferase activity with different promoter constructs:
- CP-E212-Luc
- CP-E212 Δ766-Luc
- CP-E212 Δ410-Luc
- CP-Luc

Graph showing differences in luciferase activity:
P<0.05
P<0.05
P<0.05

C

Diagram showing luciferase activity with different promoter constructs:
- CP-E212-Luc
- CP-E212 Δ766-Luc
- CP-E212 Δ410-Luc
- CP-Luc

Graph showing differences in luciferase activity:
P=n.s
P<0.05
P<0.05

C and E both had TSS corresponding to the sequence published by Yang and Polgar [23] although in their study, only the full length B1R transcript was detected (Fig 3B, transcript C). None of them corresponded to the TSS of the published B1R mRNA sequence (NM_000710.2). In addition to the alternative start sites, a novel 5’UTR SV which skips exon II of B1R was detected (Fig 3B, transcripts D and E).

Confirmation of novel 5’UTR B1R splice variant

B1R SV was detected using two different sets of common primers located in exon I (Forward primers: B1R Fow or B1R Fow 2A) and exon III (Reverse primers: B1R Rev or B1R Rev1) (Fig 4A, Table 2). The result of each primer set showed an additional band 120bp smaller than the expected wild-type B1R product indicating presence of B1R SV (Fig 4A). Sequencing of the B1R SV band confirmed the identification of the novel B1R transcript that splices out exon II exactly at the intron/exon boundary.

To determine if alternative splicing was an artefact of the immortalised cell line, H2126, RNA from other human pulmonal cell lines (16HBE, HFLF and NHLF) along with human immortalised cell line, H2126, RNA from other human pulmonary cell-lines (A549, H2126) (Fig. 1). However, the 5’UTR SV mRNA was more stable than the wild-type/full length (B1R WT) transcript (Fig. 1).

B1R 5’UTR SV transcript does not affect translational efficiency

The 5’ UTR can also play an important role in regulating the rate of mRNA translation to protein. To determine if the SV transcript affects the rate at which B1R protein is produced, the WT and 5’ UTR SV were inserted immediately upstream of the luciferase coding region and immediately downstream of a SV40 promoter. 16HBE and H2126 cells were then transfected with the constructs and luciferase activity measured over 48 hr. There was no significant difference in the rate of protein produced using either UTR construct (Fig. 6). However, there was a noted difference in the overall pattern of luciferase expression depending on cell type. In 16HBE, the luciferase protein increased gradually peaking at 48 hrs, while in H2126 it peaked at 24 hr before falling almost to basal levels by 48 hr (Fig. 6).

Effect of B1R specific stimulant DAKD on B1R WT and SV mRNA expression

B1R is an inducible gene upregulated by several inflammatory stimuli including its agonist DAKD. To determine if the WT and SV transcripts are differentially affected by DAKD, H2126 and 16HBE cells were incubated with 100 nM and 1000 nM of DAKD over a 24 hr period. B1R WT and SV gene expression (Fig. 7A & B) showed that in H2126 the WT and SV transcripts were significantly induced with 1000 nM DAKD but at different times with a peak at 3 hr for WT (increased 60%, p = 0.03) and 6 hr for SV (increased 25%, p = 0.04). In 16HBE cells WT, but not SV, mRNA expression was increased following 1000 nM DAKD stimulation for 6 hr 250%, p = 0.03) and remained elevated until 24 hr (300%, p = 0.008;Fig. 7C).

Discussion

Although kinins play an important role in airway inflammation, the regulation of the inflammation-induced kinin receptor B1R expression in pulmonary cells is unknown. We identified a novel B1R SV which is less stable than wild-type mRNA but does not appear to impact on translation efficiency. The differential constitutive and stimulated expression of SV compared to wild-type B1R suggests a role of SV in regulating B1R gene expression in human airway cells. In addition, we have identified regulatory elements, rather than a previously proposed alternative promoter, in exon II and intron II of the 5’UTR regulating the expression of B1R. These findings reveal complex regulation of B1R receptor expression which may enable its future manipulation in chronic pulmonary inflammation and cancer.
B₁R is expressed by a range of cells and tissues including the lung and is rapidly induced during inflammation [4–7]. Our group and others have reported constitutive B₁R expression in neutrophils [27], primary sensory A- and C-fibers [20], eosinophils [17], macrophages [29], dendritic cells [30,31], and pulmonary primary cells [9,10] and cell-lines [10,32,33]. In agreement with our previous work we found higher constitutive expression of B₁R in pulmonary adenocarcinoma cells (H2126, A549) and pulmonary fibroblasts (NHLF, HLF) compared to normal bronchial epithelial cells (H16BE), while squamous cell carcinoma cells (H220) did not constitutively express B₁R [34].

Following DAKD stimulation of one of the high constitutive expressors (H2126) and low constitutive expressors (16HBE) we found that the low constitutive expressor was more responsive to DAKD compared to the high constitutive expressor. Previous studies have also show high B₁R constitutive expression in a number of different cancers, including lung [35]. Further, B₁R antagonists have been efficient in inhibiting growth in a range of lung cancers NSCLC, SCLC and mesothelioma [36,37]. In contrast, low constitutive B₁R expression is upregulated in human nasal epithelial cells in allergic rhinitis subjects compared to controls [9] and in human primary bronchial epithelial cells post stimulation with IL-1β and TNF-α [10]. These differences may be explained by cell-specific regulatory mechanisms which we subsequently investigated.

Using lung fibroblast and smooth-muscle cells others have reported B₁R core and alternative promoters with the 5’ core promoter defined as 1.4 kb upstream of exon I and the alternative promoter 1020 bp upstream of exon III (intron II and exon II)[21,22]. However, there is no published evidence of a TSS driving this alternative promoter. We identified similar regulatory elements in the 5’UTR in two pulmonary cell-lines (Fig 2). The −1020 to −766 bp region of 5’UTR acts as a NRE only in high expressing H2126 cells with no effect in low expressing 16HBE. In contrast, enhancer-like elements between −1842 and −812 were previously reported in HepG2 cells [22], suggesting that this region may be responsible for cell specific activity. While this group found no further regulatory elements downstream we identified an enhancer-like element between −766 and −410 bp in the low expressing 16HBE and found that the region −410 to +1 acts as a PRE in H2126 cells and in contrast as a NRE in 16HBE cells. We demonstrated that −400 to +1 bp contains the minimum sequence required for promoter activity supported by others that report 300 bp as the minimal region [22]. We also noted that in the constructs without core promoter (data not shown), basal activity of −410 to +1 region is higher in high expressor cells than in 16-HBE low expressor cells suggesting this region may be involved in constitutive, cell-specific receptor expression.

To determine whether the 5’UTR regulatory regions could affect induction of B₁R, 16HBE and H2126 cells were stimulated with pro-inflammatory LPS and DAKD. Neither stimulus affected luciferase activity of any promoter construct. The lack of induction by LPS is in agreement with previous studies showing that LPS as well as IL-1β and TNF-α do not induce activity in human HepG2 and rat vascular smooth muscle cells [18]. The inability of DAKD to induce activity is not surprising as no consensus has been achieved from previous studies [18,19,24,38,39]. The majority of studies have failed to induce 5’ core promoter activity in human lung fibroblasts, human smooth muscle cells and peripheral blood lymphocytes [19,21,38]. This highlights the tight and delicate balance of B₁R regulation at the promoter level and is an indication that other regions outside of the promoter, exon II and intron II of B₁R, are more likely to play a role in the upregulation
of B1R by LPS and DAKD. In an attempt to locate the domains involved, Yang et al [38] constructed a human B1R minigene that consisted of 1.8 kb of the promoter, exon I, 1.5 kb of intron I, exon 2, intron 2 and luciferase gene. This minigene exhibited promoter activity with LPS and DAKD stimulation, which was abolished with the replacement of the minigene with 1.8 kb 5′ promoter construct.

To investigate whether these regulatory regions acted as a promoter in pulmonary cells we looked for alternative TSS. While we could not detect any TSS downstream of the proposed alternative promoter, we cannot exclude that this region does not act as a promoter in other cell types or in developmental stages as intronic promoters can regulate transcripts which are tissue- or differentiation-specific [40]. However, we did identify a novel kinin B1R SV, with exon II skipping, which adheres to the consensus GT-AG sequence conserved in 98% of mammalian splice sites [41,42]. This SV was detectable in a range of human immortalised pulmonary cell lines, as well as primary human leucocytes and lung tissue (data not shown). The splice variant was the dominant transcript in human leucocytes in contrast to pulmonary cell-lines.

Exon II splicing of B1R has not been documented in any other species. However, several SVs in 5′UTR have been reported in rat B1R including a 41 bp skipping at the start of the exon II which was predicted to affect translation efficiency [43–45]. Results from these and our study, suggest that 5′UTR splicing may be a common event in the regulation of kinin receptors.

Figure 4. B1R splice variant (B1R SV) transcript and heteroduplex band is present in several cell types. Amplification of B1R WT and SV using primer set RT Fow and RT Rev (A) and RT Fow2A and RT Rev 1 (B). Amplification of loading control housekeeping gene superoxide dismutase 1 (SOD1) (C). Amplification of B1R WT and SV from human leucocytes using primers RT Fow and RT Rev (D) and amplification of SOD1 (E). A representative image of additional heteroduplex band located between the WT and SV band in HFLF. Three extra PCR cycles were used to match conditions used in G, but without addition of 10X fresh PCR mix (F). Heteroduplex band removed after addition of 10X fresh PCR mix followed by 3 PCR cycles (G). Lane 1: 100 bp ladder. PCR no template control (NTC). Human leucocytes (Leuc).

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Figure 5. B1R WT is more stable than B1R SV under basal conditions. Actinomycin D (Act D) mRNA decay of B1R WT and SV transcripts in H2126 measured at 0, 1, 3, 5h using real time PCR (Act D treatment at concentration of 5 µg/mL). Data plotted is mean ± SEM from four independent experiments each performed at least in duplicates. Half-life of mRNA can be roughly estimated by determining the time required to reach 50% transcript level (shown by dotted lines). For more accurate assessment, the trendline equations obtained by plotting the graph are used to determine the half-life. In this graph, the equation for B1R WT is $y = 100e^{-0.213x}$ while for B1R SV is $y = 100e^{-0.344x}$, where y is set to 50 (indicating 50% of transcript remaining) which will allow the calculation of x (indicating time required to reach 50% transcript level). From these equations, the half-life of B1R WT is 3.28 hr and 2.02 hr for B1R SV.

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This novel human SV affects only the 5’UTR of B1R while the coding region and protein remain unchanged. 5’ untranslated regions regulate the efficiency of protein translation as well as the stability of the transcript [46]. The WT transcript half-life measured in this study was approximately 108 min and 48 min longer than the results obtained from Zhou et al and Schanstra et al respectively, who measured the half-life in human embryonic lung fibroblasts-IMR90 [7,24]. In H2126 cells, the B1R SV was ≈ 35% less stable than the wild-type B1R which may indicate a possible stabilizing element located within exon II. Characterisation of the gene structure of the human B1R suggests that exon II is part of an Alu-[ ] element that spans part of intron I, exon II and part of intron II [23]. Alu elements are small interspersed nucleotide elements present in the B1R5' UTR and the alternative splicing [47,48], initiation of translation, and translation efficiency [49–52]. More recently, the presence of Alu elements in exons and adjacent introns has been linked to forming circular RNAs, which have increasingly been reported as strong regulators of gene expression [53]. In particular, circular RNAs are cell- and developmental stage-specific post-transcriptional regulators which compete for binding by microRNAs or RNA binding proteins [54] and may contribute to the cell-specific differences in receptor expression we observed.

Our in silico analysis of B1R mRNA folding and secondary structure predicts that the B1R wild-type 5’ UTR is more stable with a free energy of −60.30 kcal/mol compared to B1R splice-variant at −16.10 kcal/mol. No discernible difference between the wild-type and SV UTR translational efficiency was observed suggesting the 5’ UTR is not involved in B1R translational efficiency. At all earlier time points including 9 hr luciferase expression was the same between both cell lines. At 24 and 48 hr there was up to a 10 fold higher luciferase expression in the lower constitutive expressing 16HBE compared to the high constitutive expressing adenocarcinoma H2126. The difference in rate of translation between 16HBE and H2126 may reflect a more active SV40 promoter in 16-HBE or the lack of B1R 3’UTR down regulatory elements in these constructs.

Cell specific differences in WT and SV expression were observed post stimulation. While in both cell lines B1R SVs were inducible, delayed upregulation of the SV transcript in H2126 cells following stimulation with the B1R-specific agonist DAKD suggests that the SV is regulated in a different manner to the WT. The profile of total B1R mRNA expression in H2126 correlates well with other studies that indicate an increase in mRNA 2–3 hr post-stimulation which is maintained at 4–6 hr and falls by 12 hr [55,56]. In 16HBE cells, the WT mRNA expression post-stimulation was highest at 24 hr. As mentioned earlier, B1R SV expression in 16HBE was undetectable. This low expression of B1R SV suggests that the SV may not be essential to the regulation of B1R expression in 16HBE. The time-dependent increase in B1R mRNA transcripts following DAKD stimulation may be due to either increased mRNA production and/or increased accumulation of mRNA due to less degradation/increased stability. The transcriptional regulatory effect of DAKD on B1R is mainly through NF-kB and AP-1 [18,24,57,58]. As both B1R transcripts arise from transcription initiated from the same TSS, it is unlikely that DAKD stimulation increases specific B1R transcripts through promoter regulation. Increasing stability of SV transcript could be a plausible mechanism whereby DAKD, either directly or indirectly, stabilizes the mRNA leading to accumulation of the SV we describe.

Conclusions

This study has identified the existence of a novel and naturally occurring SV of human B1R that reduces the length of the 5’UTR region of B1R. Characterisation of the effect of 5’UTR in terms of mRNA stability and translation efficiency revealed that the novel SV is 35% less stable than the wild-type full length transcript in H2126 cells but does not impact on the translation efficiency of the downstream protein as measured by luciferase activity. The DAKD agonist differentially increased B1R WT and SV expression in 16HBE. The time-dependent increase in B1R mRNA transcripts following DAKD stimulation may be due to either increased mRNA production and/or increased accumulation of mRNA due to less degradation/increased stability. The transcriptional regulatory effect of DAKD on B1R is mainly through NF-kB and AP-1 [18,24,57,58]. As both B1R transcripts arise from transcription initiated from the same TSS, it is unlikely that DAKD stimulation increases specific B1R transcripts through promoter regulation. Increasing stability of SV transcript could be a plausible mechanism whereby DAKD, either directly or indirectly, stabilizes the mRNA leading to accumulation of the SV we describe.
Regulation of Kinin B₁ Receptor Expression

A

H2126

Relative B₁R mRNA level

Time (hrs) of DAKD stimulation

B

H2126

Relative B₁R mRNA level

Time (hrs) of DAKD stimulation

C

16HBE

Relative B₁R mRNA level

Time (hrs) of DAKD stimulation
SV and cell specific regulatory elements within the ‘alternative promoter’. While more research is required to elucidate this observation, our findings suggest that specific targets may be available to downregulate B1R expression in inflammatory diseases in particular asthma, COPD and cancer.

Author Contributions
Conceived and designed the experiments: SB KB PJT. Performed the experiments: FY. Analyzed the data: FY. Contributed reagents/materials/analysis tools: SB KB PJT. Wrote the paper: FY SB SELT KB PJT.

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