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A role for bradykinin signaling in chronic vulvar pain

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Abstract

Chronic vulvar pain is alarmingly common in women of reproductive age and is often accompanied by psychological distress, sexual dysfunction, and a significant reduction in quality of life. Localized provoked vulvodynia (LPV) is associated with intense vulvar pain concentrated in the vulvar vestibule (area surrounding vaginal opening). To date, the origins of vulvodynia are poorly understood, and treatment for LPV manages pain symptoms, but does not resolve the root causes of disease. Until recently, no definitive disease mechanisms had been identified; our work indicates LPV has inflammatory origins, although additional studies are needed to understand LPV pain. Bradykinin signaling is one of the most potent inducers of inflammatory pain and is a candidate contributor to LPV. We report that bradykinin receptors are expressed at elevated levels in LPV patient versus healthy control vestibular fibroblasts, and patient vestibular fibroblasts produce elevated levels of proinflammatory mediators with bradykinin stimulation. Inhibiting expression of one or both bradykinin receptors significantly reduces proinflammatory mediator production. Finally, we determined that bradykinin activates NF κ B signaling (a major inflammatory pathway), while inhibition of NF κ B successfully ablates this response. These data suggest that therapeutic agents targeting bradykinin sensing and/or NF κ B may represent new, more specific options for LPV therapy.

Perspective: There is an unmet need for the development of more effective vulvodynia therapies. As we explore the mechanisms by which human vulvar fibroblasts respond to proinflammatory/pro-pain stimuli, we move closer to understanding the origins of chronic vulvar pain and identifying new therapeutic targets, knowledge which could significantly improve patient care.

Keywords

fibroblast; vulvodynia; bradykinin; cytokine; inflammation

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INTRODUCTION

Pain, while a necessary response to prevent irreparable injury, can be a perplexing condition to resolve when it occurs chronically or without obvious provocation⁷¹. Pain can affect nearly any body site or it can be generalized; the effects of chronic pain are crippling, even when the pain is not associated with any life-threatening or physically debilitating condition⁷¹. While some chronic pain conditions (e.g. fibromyalgia) have been the subject of increased public interest, others have received comparatively less attention. Among these, localized provoked vulvodynia (LPV) is a disabling, persistent, and poorly understood pain condition affecting specific regions of the vulvar vestibule (the area immediately surrounding the vaginal opening)^{30, 32, 64}.

Like many other chronic pain conditions, the origins of LPV are not clearly delineated^{32, 52, 64}. Consequently, LPV has been relegated to a “diagnosis of exclusion,” and no mechanism-based pain treatments have been developed³¹. Women with LPV experience intense pain, yet often show no obvious signs of infection, lesions, or abnormalities^{30–32, 64}. At the same time, LPV is common and affects up to 28% of women in the United States at some point during their lifetime³⁰, while roughly 8% of the population is currently afflicted^{33, 58}. Reported prevalence estimates may underestimate true prevalence, based upon the patient’s failure to seek evaluation of or the practitioner’s failure to diagnose LPV³³. The diagnostic hallmark of LPV is longstanding exquisite pain to light touching (allodynia), which is highly localized to the vulvar vestibule³². As a result, LPV leaves a devastating impact on sexuality, reproduction, self-image, and emotional wellbeing^{30, 64}.

Epidemiologic studies have identified few clinical precursors, with chronic or recurrent infection by the fungus *Candida* being reported as the most common risk factor^{1, 59, 64}. In support of these findings, repeated vulvovaginal infection with *Candida albicans* has been associated with LPV in a mouse model of disease²¹. Our recently published work also supports a *Candida*-LPV association; fibroblasts isolated from sites of LPV allodynia respond strongly to *C. albicans* and other vaginal fungal pathogens through the production of proinflammatory mediators (e.g. interleukin-6; IL-6 and prostaglandin E₂; PGE₂)^{20, 23}. We also have evidence that other inflammatory triggers (not specific to fungal infection) stimulate the production of proinflammatory mediators²⁵. IL-6 and PGE₂ have been previously associated with pain in both human and animal models^{15–17, 19, 22, 37, 45, 53, 61}. Furthermore, we have demonstrated that fibroblast production of proinflammatory mediators can accurately predict the pain threshold measured at the site of fibroblast origin²³. Therefore, like many other chronic pain conditions, LPV appears to have inflammatory origins⁷¹.

With the overarching goal of identifying new therapeutic targets, we set out to explore the mechanisms by which vestibular fibroblasts respond to proinflammatory stimuli to generate this maladaptive immune response associated with pain. Presently, therapeutics for vulvodynia manage the symptoms of LPV, but none target the underlying *inflammatory* origins of disease^{30, 32, 63}. In a previous study, we demonstrated that vestibular fibroblasts derived from LPV patients at sites with low pain thresholds express elevated levels of the

Dectin-1 receptor, which is responsible for recognizing fungal cell wall-derived β -glucan²⁰. Exposure to fungal cell wall components or fungi resulted in a potent inflammatory response, which was comparatively stronger in patient fibroblasts isolated from pain versus non-pain sites²⁰. However, blocking Dectin-1 signaling only partially ameliorated IL-6 and PGE₂ release, implying that other receptors/mechanisms contribute to proinflammatory mediator release, and likely pain²⁰. Therefore, a broader understanding of the inflammatory pathways that might affect LPV are necessary to design more effective therapeutic agents that will target the underlying biological cause(s) of disease.

Bradykinin, a nonapeptide, is one of the most potent pain-inducing molecules present within the human body^{11, 12, 48, 49, 56, 70, 72}. Bradykinin has been linked to inflammatory and neuropathic hyperalgesia⁵⁶, and inhibitors that impair bradykinin recognition have been or are currently being investigated in clinical trials as potential therapies for several painful medical conditions, such as angioedema^{5, 6, 47, 67}. Bradykinin also plays important roles in vasodilation and the oxidative stress response^{8, 56}. Kinins are produced in human plasma and specific body tissues through the cleavage of high molecular weight (HMW) kininogen precursors by plasma and tissue kallikreins, respectively⁵⁶. Kinins are highly inflammatory and can be further degraded to inactive peptides by kininases. Differential cleavage of HMW kininogens generates distinct species of kinins (e.g. Des-Arg⁹-bradykinin and bradykinin) that are recognized by two bradykinin receptors, B1 (BDKRB1) and B2 (BDKRB2), while the ligand affinity for each receptor is not equal⁵⁶. BDKRB1 is absent in most body tissues, but is readily inducible and can be detected when inflammation is present⁵⁶. BDKRB2, on the other hand, is considered to be a constitutive receptor for bradykinin and is highly expressed in all body tissues⁵⁶, although several studies show that it can be further induced under inflammatory conditions^{14, 44, 60}.

We became interested in bradykinin because of its inflammatory and pain-inducing qualities⁵⁶ and the observation that *Candida*, which has been implicated in vulvar pain, may play a role in exacerbating the responses to bradykinin by generating locally elevated concentrations of bradykinin^{9, 10, 35, 36, 39, 57}. *Candida* species produce serine aspartyl proteases that cleave human HMW kininogens, which is believed vital to pathogenesis and aids in tissue invasion by *C. albicans*^{9, 10, 35, 39, 57}. Therefore, we hypothesized that bradykinin may also play important roles in inflammation and pain within the vulvar vestibule. To evaluate our hypothesis, we first examined whether vestibular and external vulvar fibroblasts express the machinery required for bradykinin processing and sensation, then evaluated the influence of bradykinin on proinflammatory mediator release (IL-6). Furthermore, we investigated the mechanism by which bradykinin is sensed in pain-associated vestibular fibroblasts.

MATERIALS AND METHODS

Patient/Sample Selection.

LPV-afflicted cases (fulfilling Friedrich's Criteria⁷) and age/race-matched pain-free controls were recruited from the Division of General Obstetrics and Gynecology clinical practice at the University of Rochester between December 2012 and February 2014. All subjects provided informed consent, and the research was approved by the University of Rochester

Institutional Review Board (RSRB # 42136). Expanded details on our selection criteria and sampling procedures have been previously published^{23, 25}. In brief, cases and controls were age- and race-matched with a mean age of 33.5 years for both groups. All case and control subjects were Caucasian, non-Hispanic. Furthermore, all subjects denied the use of corticosteroids and non-steroidal anti-inflammatory medications and had no chronic inflammatory illnesses other than LPV. Prior to biopsy of the vestibular and external vulvar sites, sampling sites underwent mechanical Wagner™ algometry. The Wagner™ algometer method (Wagner Instruments, Greenwich, CT) used a Method of Limits technique initially described by Zolnoun et al.⁷³ and further elaborated on in our earlier publication²³. Using the Wagner™ algometer, an increasing 0.5 N per second force (range 0 to 5 N) was applied perpendicular to the mucocutaneous surface by a moistened dacron tipped swab affixed to the Wagner™ algometer. Force was terminated at the point of pain development (singaled by hand-held clicker) or when the test reached 5 N force. Tissue was sampled for fibroblast strain development from sites as diagrammed previously^{23, 25}. A total of 3 paired case (vulvar vestibule and external vulva) and 3 paired control fibroblast strains (12 total) were used in this study, which were also used in a preceeding study²⁰.

Fibroblast strains.

Primary fibroblast strains (each obtained from a different patient or healthy control) were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, GlutaMAX, gentamycin, and antibiotic/antimycotic solution (Gibco/Invitrogen/Thermo Fisher Scientific, Grand Island, NY) as previously described⁴. Early passage (4–10) external vulvar and vestibular fibroblast strains were seeded at 2.5×10^4 cells/cm². After cultures reached full confluence, fibroblasts were serum-reduced for 48 h in MEM supplemented with 0.05% FBS. Fibroblast cellular identity was confirmed by microscopic inspection and with fibroblast-specific markers (e.g. vimentin, collagen). At the same time, the cells were confirmed to be negative for epithelial cell markers (e.g. cytokeratin), smooth muscle and myofibroblast markers (e.g. α -smooth muscle actin), endothelial cell markers (e.g. CD34), and bone marrow derived cell markers (e.g. CD45)³⁸.

Transcriptomic analysis of fibroblasts.

A comparative transcriptome analysis was performed on vestibular versus external vulvar fibroblasts, representing selected pathophysiologic areas of interest. These included pain-associated factors, pro-inflammatory factors, innate immunity factors, neurotropic/growth factors, and homeobox sequences. Fibroblast strains from the vestibule and external vulva of a selected LPV case, which had previously undergone cytokine expression analysis, were selected for this comparative study. Total RNA from vestibular and vulvar fibroblasts was harvested using the miRNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. After RNA isolation, a total RNAseq library was generated using a TruSeq RNA sample kit (Illumina, San Diego, CA), and RNAseq was performed using an Illumina HiSeq2500 Sequencer at the University of Rochester Genomics Research Center. FastQC (version 0.10.0) was used to run preliminary QC analysis on all samples. RNA-Seq unified mapper (RUM v1.11) was used to align the short reads to the human genome. The RUM algorithm is both accurate and rapid compared to other popular aligners for next-generation

sequencing²⁹. Sequencing and analysis were performed at the University of Rochester Functional Genomic Core.

Quantitative real-time PCR (RT-qPCR).

Expression profiles of the bradykinin receptor 1 (BDKRB1) and bradykinin receptor 2 (BDKRB2) mRNA sequences were evaluated after being treated with 1 μ M bradykinin (Sigma-Aldrich, St. Louis, MO) or vehicle control for 24 h in fibroblast strains obtained from LPV cases and controls. Cells were propagated and treated in 24-well plates. At 24 h, cells were lysed and total mRNA was extracted using the Qiagen RNeasy kit following the manufacturers' instructions (Qiagen Corp., Carlsbad, CA). A NanoDrop ND-1000 (NanoDrop/Thermo Fisher Scientific) was used to quantify the mRNAs, which were used as templates for cDNA synthesis using the iScript cDNA synthesis kit (BioRad, Hercules, CA); 50 ng total RNA template was used in each reaction. Negative reverse transcriptase controls (where no enzyme was added to the reaction) were also prepared to confirm the absence of DNA contamination. cDNA samples were diluted 5-fold in RNase-free molecular grade water (Qiagen) and used as templates for qRT-PCR reactions (5 μ l/reaction). A standard curve was constructed for each primer set by preparing 5-fold serial dilutions of a reference set of cDNAs prepared from RNAs purified from cells treated with zymosan (Sigma-Aldrich). Reactions were prepared in a total of 12 μ l using SsoAdvanced Universal SYBR Green Supermix (BioRad). Primers (sense 5'-AGTGGCACAATCATAGCTCG and antisense 5'-CCGTAGCATTTTGAGGGAAGAG) were designed for human BDKRB1 using IDT oligo design tools (OligoAnalyzer, <http://www.idtdna.com>), while published primer sequences for human BDKRB2⁴⁰ were used to quantify bradykinin receptor expression. All RT-qPCR values were normalized to the 18S rRNA signal amplified using previously published primer sequences⁴³. Each experiment was performed in quadruplicate.

Bradykinin receptor protein expression on human external vulvar and vestibular fibroblasts.

Fibroblast strains (external vulvar and vestibular strains from both cases and controls) were grown to confluency in 6-well culture dishes (Greiner Bio-One, Monroe, NC) and treated with 1 μ M bradykinin or vehicle control for 24 h. Cells were then released from the culture surface using trypsin-EDTA solution and trypsin inhibitor (Thermo Fisher Scientific), and washed with PBS before blocking non-specific antibody binding with 5% human Fc receptor blocker (Miltenyi Biotech Inc., San Diego, CA) in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. Cells were then either left unstained or incubated with AlexaFluor488 (AF488)-conjugated anti-human BDKRB1 antibody or AlexaFluor647 (AF647)-conjugated anti-human BDKRB2 antibody (both from GeneTex Inc., Irvine, CA) for 30 min at 4°C. Cells were analyzed on a FACS Canto II flow cytometer running FACSDIVA software (BD Biosciences, San Jose, CA) using a 488 nm excitation laser and a 530/30 nm band pass detector filter for AF488 or a 633 nm excitation laser with a 660/20 nm band pass detector filter for AF647. Subsequent analysis of fluorescence emission data was performed using FlowJo software (version 10.1; FlowJo LLC, Ashland, OR). A representative result from several flow experiments is depicted.

Additional 6-well cultures of fibroblast strains were prepared as described earlier, then washed with PBS, and lysed in 0.1 M Tris containing 2% sodium dodecyl sulfide (SDS) and 1/10 the volume of protease inhibitor cocktail (Sigma-Aldrich). Total protein concentrations were determined using a BioRad DC protein assay, and 5 µg of each protein lysate was run on a 10% SDS-polyacrylamide (PAGE) gel with Spectra multicolor broad range protein ladder (Thermo Fisher Scientific) and electro-transferred to a 0.45 µm EMD Millipore Immobilon PVDF membrane (Thermo Fisher Scientific). Membranes were stained with Ponceau S (Sigma-Aldrich) for 10 min to visualize total protein on the membrane. Membranes were de-stained in 5% acetic acid, then washed in western wash buffer (PBS with 0.1% Tween-20) several times before blocking with 2% bovine serum albumin for 30 min (reagents from Sigma-Aldrich). After blocking, membranes were incubated with a goat polyclonal antibody specific for BDKRB1 (Genetex), a rabbit polyclonal antibody specific for BDKRB2 (Genetex) or rabbit polyclonal antibody for β -tubulin (loading control; Cell Signaling Technologies, Danvers, MA) for 1 h at room temperature (RT). Membranes were washed and then incubated with a horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) or HRP-conjugated mouse anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. BDKRB1 and BDKRB2 receptor expression was visualized using enhanced chemiluminescent HRP substrate (Thermo Fisher Scientific) and exposure to x-ray film, followed by densitometric analysis using Quantity One 1-D Analysis Software version 4.6.9 (BioRad).

Bradykinin dose response.

Cultures of fibroblast strains were seeded to 24-well tissue culture plates at roughly 50% confluence and were allowed to grow until confluent (~3–4 days) at 37°C and 5% CO₂ in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), GlutaMAX, gentamicin, and antibiotic/antimycotic solution (Thermo Fisher). Once confluent, cells were transitioned to serum-reduced media (supplemented with 0.05% FBS) and incubated for 48 h. Confluent fibroblast wells were then treated with a dose range of bradykinin (0.001–10 µM, based on concentrations used in previous studies¹³) or vehicle control and incubated for 24 h at 37°C and 5% CO₂. A standard sandwich ELISA was used to measure the production of IL-6 (BD Biosciences, Franklin Lakes, NJ). Experiments were performed a minimum of two times in quadruplicate.

Bradykinin receptor knockdown.

The impact of BDKRB1, BDKRB2, or simultaneous BDKRB1 and BDKRB2 inhibition was evaluated by quantifying the amount of IL-6 released in response to treatment with 0.1 µM bradykinin (lowest dose that elicits a substantial proinflammatory response) in cells with functional receptor versus those with impaired receptor function. IL-6 was assayed because it is abundantly produced by fibroblast strains from LPV cases^{23, 25} and has been generally associated with the evolution of pain during inflammation^{17, 19, 37, 53, 66}. Furthermore, bradykinin has been shown to induce the production of IL-6 by other researchers¹² and from our fibroblasts strains (this study). Cells were grown in 24-well plates in MEM with 10% FBS until confluent, then transitioned to low serum media (0.05% FBS) for 48 h. We used a molecular approach to block the expression of the genes encoding BDKRB1 and BDKRB2.

Small-interfering RNAs (siRNAs) against human BDKRB1 and BDKRB2 and Silencer negative control no. 1 siRNA were purchased from Ambion (a division of Thermo Fisher Scientific). The Lipofectamine 2000 reagent (Ambion) was used to transfect cells with control and anti-bradykinin receptor siRNAs according to the manufacturer's instructions; cells were first washed with PBS, then transfected with a total of 500 ng siRNA/well in MEM with 0.05% FBS. Following the transfection procedure, cells were incubated for 48 h at 37°C. At this time, cells were then challenged with 0.1 μ M bradykinin or vehicle control in fresh media for another 24 h, at which point supernatants were collected for detection of IL-6. The adherent cells were then washed in PBS, and protein was collected for Western blotting against BDKRB1 and BDKRB2. Each experiment was performed a minimum of two times in quadruplicate.

Bradykinin receptor inhibition

Cells were grown in 24-well plates in MEM with 10% FBS until confluent, then transitioned to low serum media (0.05% FBS) for 48 h. Cells were pre-treated with 5 μ M R715 (Tocris Bioscience Avonmouth, Bristol, United Kingdom) or 1 μ M HOE-140 (Sigma-Aldrich) for 1 h prior to treatment with 0.1 μ M bradykinin or vehicle control for 24 h. After 24 h, we measured the amount of IL-6 produced using our standard sandwich ELISA. Each experiment was performed a minimum of two times in quadruplicate.

Activation of NF κ B with bradykinin challenge.

To assess the effect of bradykinin on the activation of the NF κ B pathway, LPV patient fibroblast strains were transfected with an NF κ B-firefly luciferase reporter³ and pRL-SV40, a commercially available control renilla luciferase reporter (Promega, Madison, WI). Nucleofection of these constructs was performed as previously described³. Cells were allowed to reach confluence in 24-well plates, then serum-reduced 48 h prior to transfection. After transfection, cells were allowed to recover for 24 h before transitioning to fresh media (MEM + 0.05% FBS) containing 0.1 μ M bradykinin or vehicle control. After 24 h of treatment, reporter activity was determined using the Dual-Glo Luciferase Assay System (Promega, Madison, WI). Luminescence was quantified on a VarioSkan Flash Multimode Reader (Thermo Fisher Scientific). Firefly luciferase activity was then normalized to constitutive renilla activity to adjust for any differences in the final numbers of cells in each well. The same protocol was performed using 5 ng/ μ l IL-1 β , a strong inducer of NF κ B signaling, as the agonist. Assays were performed a minimum of two times in quadruplicate.

Impact of NF κ B inhibition on pro-inflammatory mediator release in response to bradykinin.

Patient fibroblast strains were cultured in 24-well plates as described and then simultaneously treated with either bradykinin alone or with 0.1 μ M bradykinin and 5 μ g/ml BAY-11-7082 (NF κ B inhibitor; Cayman Chemical) in MEM + 0.05% FBS. Cells were incubated for 24 h at 37°C prior to supernatant collection. The amount of IL-6 in the supernatant was determined by sandwich ELISA as detailed earlier. Assays were performed a minimum of two times in quadruplicate.

Statistical analysis

GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) was used to conduct the statistical analysis. Paired t-tests were used to compare between vestibular and external vulvar cells, cases and controls, or treated and vehicle control samples. Significance was set at $P = 0.05$ for a two-tailed distribution. A multiple linear regression analysis was performed including two independent variables: presence of disease and the post-bradykinin stimulated IL-6 production, and one dependent variable: mechanical pain threshold following natural log transformation. This model was developed to ascertain whether post-bradykinin IL-6 production by location specific fibroblasts could predict pain level. Heteroskedasticity and collinearity were ruled out by inspection of residuals, Cook-Weisberg test, and pairwise correlation. The scatterplot, estimated regression of the model and 95% confidence intervals were also graphically presented. The data are expressed at the mean $\pm$ the standard deviation.

RESULTS

Vulvar fibroblasts encode the machinery to detect bradykinin.

Our first step in evaluating whether vulvar fibroblasts encode the machinery for responding to bradykinin was to evaluate global gene expression using RNAseq. Comparing gene expression between vestibular and external vulvar fibroblast strains isolated from a prototypical patient revealed that both cell types express genes implicated in kininogen processing and kinin sensing (Table 1). Each strain likely encodes enzymes for processing plasma and tissue kininogens (termed kallikreins), while the number of tissue kallikreins detected in pain-associated vestibular fibroblasts may be higher than in external vulvar fibroblasts. Of note, neither cell type encodes the high molecular weight (HMW) kininogen precursor, which suggests that these cells can process, but do not generate HMW kininogens. Both cell types robustly expressed bradykinin receptors (BDKRB1 and BDKRB2), while vestibular fibroblasts expressed these receptors at significantly elevated levels compared to external "pain-free" vulvar fibroblasts ($p < 0.05$). Values below 0.1 fragments per kilobase of exon per million fragments (FPKM) denote infrequent detection of the transcript that may not be significantly above background. Nonetheless, the strong expression of BDKRB1 and BDKRB2 along with the potential expression of other members of the bradykinin signaling pathway suggests this system is present in vulvar fibroblasts.

To confirm our RNAseq results, we used RT-qPCR to further evaluate mRNA expression of the bradykinin receptors that were identified as significantly differentially expressed via RNAseq after a 24 h treatment with bradykinin or vehicle control. In a larger survey of vestibular and external vulvar fibroblasts strains, which included both LPV cases and controls, we found that all strains express these receptors, while the pattern of expression varies among strains and is influenced by bradykinin stimulation (Figure 1). In our previous work, we demonstrated that vestibular expression of another receptor (Dectin-1) implicated in allodynia is often elevated in vestibular versus vulvar fibroblasts²⁰. Therefore, we compared the ratios of vestibular over external vulvar fibroblast receptor expression for each individual. We found that for BDKRB1, the vestibular:vulvar ratio for normal controls is near 1 (equal vestibular and vulvar expression) and does not change significantly with

bradykinin treatment, although treatment with bradykinin enhances receptor expression in each cell type versus its respective baseline level of expression. However, in LPV cases, baseline levels of BDKRB1 are elevated in vestibular cells, and bradykinin treatment augments vestibular expression, further increasing the ratio of vestibular:vulvar expression. For BDKRB2, the expression ratio is near one for all strains prior to bradykinin treatment, while bradykinin treatment increases the ratio in LPV cases only. These data suggest that the abundance of the bradykinin receptors is highest in vestibular fibroblasts taken from LPV cases, while treatment with bradykinin can further induce vestibular expression. Elevated vestibular expression could increase the relative sensitivity to bradykinin and therefore augment the pain response in those cells.

The BDKRB1 and BDKRB2 receptors are expressed on the surface of vulvar fibroblasts and exhibit case and site specific differences in abundance.

To examine the protein abundance of bradykinin receptors in vulvar fibroblasts, we first analyzed BDKRB1 and BDKRB2 surface expression via flow cytometry; we compared LPV case and normal control strains, both with and without bradykinin stimulation (Figure 2). For both receptors, we found that surface expression was greater in LPV case fibroblasts than in normal control fibroblasts at baseline (vehicle control), and with bradykinin stimulation (Figure 2A–D). In most situations, the mean fluorescence intensity (MFI) was slightly higher in vestibular versus external vulvar strains and increased with bradykinin treatment (Figure 2E). These results indicate that both receptors are present in all strains tested, although BDKRB1 and BDKRB2 appear to be more abundant in LPV cases versus controls and in vestibular versus external vulvar cells. Furthermore, bradykinin treatment may augment receptor protein expression, which could amplify the response to bradykinin.

We also examined protein expression by Western blotting with antibodies specific for BDKRB1 and BDKRB2, which were normalized to a β -tubulin control. While BDKRB1 and BDKRB2 were expressed in all strains, the expression of both receptors was significantly higher in LPV cases than in pain-free controls (Figure 3). For BDKRB2 in particular, not only was expression elevated in LPV cases versus controls, but it was also significantly more highly expressed in vestibular versus external vulvar fibroblasts. This was true of both paired LPV case and control strains. Altogether, elevated expression of bradykinin receptors in LPV patients at sites of pain may help to account for the heightened pain associated with LPV. In patients, pain is localized to the vulvar vestibule where the bradykinin receptors are most highly expressed.

Vulvar fibroblasts respond to bradykinin in a dose-dependent manner.

To assess whether bradykinin signaling contributes to the production of pain-associated proinflammatory mediators, we measured the amount of IL-6 produced in response to treatment with bradykinin over a dose range of 0.001–10 μ M. IL-6 has been linked to allodynia in human and animal models^{16, 19, 22, 53}, and we have shown that IL-6 levels are inversely correlated with pain thresholds in women with LPV (lower pain thresholds denote more intense pain in response to touch)²³. Therefore, IL-6 was used as sentinel indicator of proinflammatory/pro-pain signaling (Figure 4). We found that bradykinin significantly induces IL-6 (over vehicle control) at doses of 0.01 μ M and above. This is consistent with

studies in other human cell strains; even nanomolar concentrations of bradykinin can elicit a strong proinflammatory response¹³. We elected to use a dose of 0.1 μ M for subsequent experiments, as this was the lowest dose that elicited a robust response.

When comparing cases and controls, we also found that LPV cases responded more strongly to identical doses of bradykinin, generating roughly twice as much IL-6. Pain-associated vestibular cells also tended to show a trend of higher IL-6 than their external vulvar counterparts (with the exception of normal control 1; Figure 4A), but this finding was not statistically significant. Overall, this response pattern fits with the receptor abundance data; cells producing the highest levels of IL-6 also have the highest levels of BDKRB1 and BDKRB2 expression (Figures 1–3). IL-6 is associated with LPV pain²³ and when treated with very low doses of bradykinin, fibroblasts from LPV cases and from highly painful vestibular areas most abundantly produce IL-6. Therefore, these data suggest that the detection of bradykinin may be an additional mechanism that contributes to LPV pain, which may complement the published mechanism implicating Dectin-1 signaling in vulvodynia²⁰.

Post-bradykinin stimulated fibroblast IL-6 production predicts mechanical pain threshold.

To determine if bradykinin signaling is correlated with LPV pain, we assessed fibroblast strain IL-6 production following treatment with 0.1 μ M bradykinin for all sampled sites ($n = 8$ strains). A linear regression model demonstrated that IL-6 production following bradykinin stimulus predicted the pain threshold ($t = -2.91$; $P = 0.03$), adjusting for the presence or absence of LPV (Figure 5). The developed regression model predicted 91% of the variance in pain threshold levels. These data implicate bradykinin signaling in vulvar allodynia, the defining characteristic of LPV.

Inhibition of bradykinin receptor expression with siRNA significantly reduces proinflammatory IL-6 signaling.

As it is possible that bradykinin could be detected by receptors other than BDKRB1 and BDKRB2, we used siRNA to inhibit the expression of one or both of these receptors to assess their relative contribution to IL-6 signaling under bradykinin stimulation (Figure 6). We elected to use siRNA, because this approach allows for inhibition of each receptor individually or together. Inhibitors for either BDKRB1 or BDKRB2 are selective, which means they enhanced specificity for a single receptor, yet target both BDKRB1 and BDKRB2^{5, 6, 47, 67}. Our first step was to confirm successful inhibition of BDKRB1 and BDKRB2 protein expression using Western blotting, which was normalized to β -tubulin. We found that siRNA against either receptor resulted in a greater than 90% reduction in protein expression (Figure 6A).

We next compared the impact of reduced expression of the BDKRB1 and BDKRB2 proteins on IL-6 production in vestibular and external vulvar fibroblasts treated with 0.1 μ M bradykinin (Figure 6B–C). In keeping with the data obtained from the bradykinin dose response, we found that the LPV case produced elevated levels of IL-6 compared to the normal control when each was challenged with bradykinin. For both the LPV case and control, we found that reduced expression of either BDKRB1 or BDKRB2 resulted in a

statistically significant decrease in the amount of IL-6 produced by vestibular fibroblasts, while there were no significant differences in expression among external vulvar fibroblasts. However, external vulvar fibroblasts responded poorly to bradykinin overall; these values were not significantly higher than the background levels of IL-6 in vehicle-treated cells (data not shown). In the LPV case, there was a greater reduction in IL-6 when both receptors were simultaneously inhibited compared to inhibition of either receptor alone. However, simultaneous inhibition of these receptors in the control did not further dampen IL-6 release, which may be related to the overall lesser response observed in normal controls. At the same time, inhibition of either one or both receptors does not reduce IL-6 production to background levels in any of the strains tested. Treatment with control or bradykinin receptor-specific siRNA in the absence of bradykinin did not induce IL-6 production (data not shown), indicating that siRNA transfection itself does not generate a proinflammatory response in this system.

Because siRNA inhibition of the BDK receptors had a significant, but not dramatic impact on IL-6 production, we elected to try inhibition with the selective chemical inhibitors, R715 (selective for BDKRB1) and HOE-140 (selective for BDKR2). We found that both inhibitors, when used to pre-treat cells, significantly reduced the amount of IL-6 produced by pain-associated vestibular fibroblasts (Figure 7). The magnitude of reduction was similar to that observed with siRNA, supporting our earlier findings. These data suggest that while other receptors are likely involved in the bradykinin response and IL-6 release, BDKRB1 and BDKRB2 receptors play a crucial role in bradykinin signaling and IL-6 release.

Exogenous bradykinin activates the NF κ B proinflammatory pathway.

IL-6 production is driven by NF κ B activation⁴¹, and our previous work shows that NF κ B signaling is turned on in response to live fungi or zymosan treatment, both of which have been associated with proinflammatory mediator production in LPV fibroblasts²⁰. Therefore, we wanted to determine whether bradykinin signaling was another mechanism by which the proinflammatory activities of the NF κ B pathway are initiated. To assess bradykinin-mediated activation of NF κ B, we used an NF κ B reporter construct that produces luciferase when NF κ B-mediated transcription is activated. Importantly, we used primary LPV strains to measure endogenous NF κ B activity; while these strains are hard to transfect, the results more accurately reflect in vitro activation. We found that treatment with bradykinin results in a significant induction in NF κ B luciferase activity compared to a vehicle-treated control (Figure 8A). Although there were no statistically significant differences between the LPV case and control or vestibular and external vulvar cells, all showed a significant induction over vehicle (denoted by a fold-change over vehicle of greater than one). Overall, vestibular cells appeared to be more highly activated, although this observation did not achieve statistical significance. Furthermore, we compared NF κ B activation by bradykinin to activation by interleukin-1 beta (IL-1 β), a known strong inducer of NF κ B signaling (Figure 8B)⁴¹. We found that treatment with bradykinin activated NF κ B signaling roughly as potently as IL-1 β ; there were no significant differences in activation in bradykinin versus IL-1 β -treated fibroblasts. These data implicate a role for bradykinin in NF κ B activation, which would increase the production of IL-6 and cause pain.

Inhibition of the NF κ B pathway abrogates IL-6 signaling in bradykinin-treated vulvar fibroblasts.

Currently, therapeutic approaches for LPV only manage symptoms but do not address the root cause(s) of vulvodynia^{30, 32, 64}. Our previous work has shown that suppressing NF κ B signaling can successfully ablate IL-6 production²⁰, which might represent a possible therapeutic approach. However, it is unclear whether this might also be a successful approach for reducing the response to other relevant stimuli, such as bradykinin. Therefore, we tested the ability of BAY-11-7082, a commercially available and widely used inhibitor of the NF κ B pathway, to reduce IL-6 production in response to 0.1 μ M bradykinin stimulation (Figure 8C–D). We found that for two independent LPV cases, inhibition of NF κ B completely abrogated IL-6 release in both vestibular and external vulvar cells, indicating that NF κ B signaling may represent a primary means by which vulvar cells respond to bradykinin. Ultimately, inhibiting NF κ B successfully quenches the proinflammatory response induced by bradykinin (measured in terms of IL-6 production).

DISCUSSION

Although the pathogenesis of LPV remains unidentified, a number of co-morbid conditions are associated with LPV symptoms, including recurrent fungal vulvovaginitis, a history of sexual or physical abuse, contact dermatitis, fibromyalgia, interstitial cystitis, surgical trauma or traumatic childbirth, pelvic floor dysfunction, gene polymorphisms, and inflammation^{2,18, 20, 21, 23, 26, 28, 42, 46, 65, 68, 69}. Of these potential triggers, increasing evidence points to inflammation as a predominant instigator of vulvodynia^{20, 23–25, 42, 65, 68}. Inflammation is also a common catalyst in many other chronic pain conditions that lack discernable physical causes⁷¹. Our recent work has demonstrated that fibroblasts isolated from sites of pain within the vulvar vestibule are hyper-responsive to several inflammatory triggers, thus generating a detrimental proinflammatory response that is characterized by elevated production/release of IL-6, PGE₂, and likely other proinflammatory mediators linked to pain^{20, 23}. We have gathered new evidence that inflammation might be a precipitating factor for LPV^{20, 23}. Here, we implicate bradykinin, a potent inducer of inflammatory pain, in the generation of this maladaptive proinflammatory response associated with (previously unexplained) vulvar pain.

Bradykinin-related peptides, or kinins, are involved in nearly all types of inflammation and are clearly linked to pain phenotypes¹⁰. During inflammation, BDKRB1 and BDKRB2 expression is elevated and the local concentrations of their kinin agonists increase, although the precise concentrations of bradykinin at specific sites within the human body are largely unknown⁵⁶. At the same time, kinins induce the production of proinflammatory mediators, including nitric oxide, prostaglandins, and cytokines (e.g. IL-6)¹⁰, some of which are directly implicated in pain (e.g. PGE₂ and IL-6)^{15–17, 19, 22, 23, 37, 45, 53, 61}. For this reason, we elected to examine the influence of bradykinin on proinflammatory mediator production in LPV strains. Here, we have shown that vulvar fibroblasts taken from areas of pain (vestibule) and non-painful areas (external vulva) both express the machinery necessary to process HMW kininogen precursors and respond to their kinin metabolites, indicating that this system is active in these cells. Although bradykinin-sensing systems have been localized

to the mammalian gastrointestinal tract²⁷, they not been previously reported in the human vulvar mesenchyme. As discussed in our earlier paper²⁰, the urethra and vulvar vestibule in the human embryo arises from the cloacal membrane, a hindgut derivative^{54, 55}. Therefore, bradykinin-sensing, found in colonic tissue, urethra, and bladder, might be expected to be found in the vulvar vestibule as well⁶². Further exploration into the expression patterns of BDKRB1 and BDKRB2 revealed that their mRNA and protein levels correlate with one another, but differ among vulvar fibroblast strains. Specifically, we found that these receptors were most highly expressed in strains obtained from painful sites within the vulvar vestibule of LPV patients and that expression was further induced by treatment with bradykinin. This pattern of expression is similar to that observed for Dectin-1, a receptor previously implicated in LPV pain²⁰. While we have not directly tested the impact of elevated receptor expression in these areas, it would stand to reason that elevated receptor expression may help to account for elevated proinflammatory mediator production by these cells.

Although elevated expression of BDKRB1 and BDKRB2 in pain-associated fibroblasts suggested that these receptors play a role in LPV, additional evidence was necessary to evaluate their role in proinflammatory/pro-pain mediator production. To this end, we found that treatment with exogenous bradykinin resulted in strong IL-6 induction, even at low nanomolar concentrations. Strikingly, we found that the amount of IL-6 produced was higher in LPV cases than in normal controls (~2-fold), as well as generally higher in vestibular versus external vulvar cells, although not statistically significant. Therefore, IL-6 correlates with BDKRB1 and BDKRB2 expression; cells expressing elevated levels of receptor produce greater amounts of IL-6. To confirm the role of BDKRB1 and BDKRB2 in proinflammatory signaling, we evaluated the impact of siRNA receptor knockdowns on IL-6 and found that reduced expression (>90% knockdown) of either receptor resulted in a significant reduction in IL-6, while simultaneous knockdown of both receptors further reduced IL-6 (in cases only). Again, fibroblasts from LPV cases produced elevated levels of IL-6 compared to fibroblasts from normal controls, supporting the hypothesis that bradykinin is linked to LPV pain. Due to its association with IL-6 release and the link between IL-6 and LPV pain, inhibiting bradykinin sensing might be a possible target for new LPV therapeutics. At present, several bradykinin receptor inhibitors are currently being evaluated in clinical trials, which could lead to a faster clinical translation^{4, 5, 39, 54}.

However, bradykinin receptor inhibition does not completely reduce IL-6 to background levels, suggesting that other receptors/mechanisms may be involved in the response to bradykinin²⁰. BDKRB1 and BDKRB2 are regarded as the paradigm kinin receptors, which belong to the G protein-coupled receptor (GPCR) family⁵⁶. However, other uncharacterized GPCRs with homology to BDKRB1/2 may be able to recognize bradykinin. It is also plausible that the residual levels of BDKRB1 and BDKRB2 remaining after siRNA knockdown are sufficient to potentiate a response, especially considering that bradykinin's effects are manifold⁵⁶. For example, bradykinin can elicit arachidonic acid release and in turn initiate prostanoid production, which has potent effects on cellular signaling⁵⁶. Furthermore, bradykinin can activate the transient receptor potential vanilloid receptor type 1 (TRPV1) ion channel, which alters calcium/sodium ion influx and cellular signaling and has been linked to several pain conditions⁵⁶.

In addition, several studies have implicated a role for bradykinin signaling in NF κ B activation⁵⁶, which is a key inflammatory pathway linked to the production of pain-associated proinflammatory mediators. Previously, we demonstrated that Dectin-1 signals through NF κ B and that while inhibiting Dectin-1 only reduces IL-6 and PGE₂ release, inhibiting NF κ B successfully ablates proinflammatory mediator release²⁰. Here, we show that bradykinin also strongly induces NF κ B activation, and inhibiting NF κ B completely blocks IL-6 production. These results are similar to those obtained from cells activated with either live fungi or fungal cell wall components²⁰, suggesting that NF κ B activation may be a central response to challenge with various inflammatory stimuli encountered within the vulvar vestibule. Furthermore, NF κ B appears to be a chief inflammatory regulator in external vulvar and vestibular fibroblasts, which may represent a new therapeutic target for LPV. However, due to the essential nature of NF κ B in most inflammatory processes⁴¹, the ramifications of targeting this pathway could include impaired host defense at a site (the vulva) that is continuously exposed to microbial insults^{50, 51}. Nonetheless, as we gather additional information on the inflammatory pathways that influence LPV and how they are connected, the probability increases that a more ideal therapeutic target will be identified. Further elucidation of the NF κ B signaling pathway in vestibular fibroblasts may reveal more suitable targets for therapeutic intervention, as knockdown of any of the three receptors (Dectin-1, BDKRB1/2) thus far identified as playing a crucial role in vulvodynia does not completely alleviate inflammation, and globally targeting NF κ B may be problematic.

Through a series of studies^{20, 23, 25}, we have been testing the premise that the vulvar vestibule of all women possesses a unique, embryologically-defined, inflammatory/immunologic responsiveness, while LPV pain reflects an extreme example of a natural phenomenon. In this study, we have identified a new mechanism involved in chronic vulvar pain that could be targeted to treat LPV. In summary, this new mechanism involves the heightened expression of bradykinin receptors on the surface of vestibular fibroblasts obtained from sites of intense pain in LPV patients. Vulvar fibroblasts respond vigorously to bradykinin, producing copious amount of IL-6, the levels of which correlate with receptor expression patterns and are elevated in fibroblasts taken from painful areas. IL-6 production is linked to numerous pain conditions^{16, 19, 22, 53}, and there is a direct correlation between yeast-stimulated IL-6 release from human vulvar fibroblasts and LPV pain²³. Here, we show that bradykinin-induced IL-6 production is also linked to LPV pain. Although targeting bradykinin receptors does not completely resolve this detrimental inflammatory response, the response to bradykinin is tied to NF κ B signaling. Bradykinin induces NF κ B signaling at a magnitude similar to an established strong inducer (IL-1 β) of NF κ B, while inhibition of NF κ B ablates the response to bradykinin. Thus, NF κ B would likely be an effective therapeutic target. Furthermore, topical application would reduce the risk of any undesirable systemic effects that may accompany LPV treatment with agents targeting NF κ B³⁴.

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Highlights:

- Human vulvar fibroblasts respond to bradykinin, linked to inflammation and pain.
- Fibroblasts sampled from sites of allodynia have heightened bradykinin sensitivity.
- Bradykinin elicits production of algogenic factors by vulvar cells.
- Impairing bradykinin sensing reduces algogenic signaling.
- Dampening bradykinin signaling may have therapeutic benefits for LPV.

Figure 1. Vulvar and vestibular fibroblasts from cases and controls express bradykinin receptor (BDKR) mRNAs.

Relative expression of the BDKRB1 (panel A) and BDKRB2 (panel B) receptors was assessed via RT-qPCR in case and control fibroblast strains. All values were normalized to an 18S control, and expression is plotted as the ratio of vestibular over external vulvar expression. Ratios greater than one denote higher expression in vestibular versus external vulvar cells. Cells were either treated with vehicle control (no stimulus; light bars) or 1 μ M bradykinin (dark bars). BDKRB1 expression was nearly equivalent in control vestibular and external vulvar cells, while baseline (vehicle-treated) expression was higher in the vestibular

cells taken from cases; treatment with bradykinin did not dramatically alter expression in the controls, while it induced relatively higher expression in the vestibular cells from at least one case. BDKRB2 receptor expression was nearly equivalent in controls both with and without bradykinin treatment, while expression in the cases was nearly equivalent prior to treatment, but increased in vestibular cells following bradykinin treatment. All cells express both receptors, while expression levels are comparatively higher in vestibular fibroblasts cultured from LPV patients.

Normal Control Fibroblasts

LPV Case Fibroblasts

E

Mean Fluorescence Intensity (MFI)		BDKRB1	BDKRB2
Normal Control	Unstained	382	73
	Vestibular + Vehicle	483	3711
	Vestibular + Bradykinin	597	6869
	Vulvar + Vehicle	446	8106
	Vulvar + Bradykinin	538	10190
LPV Case	Unstained	143	31
	Vestibular + Vehicle	239	4356
	Vestibular + Bradykinin	342	7090
	Vulvar + Vehicle	232	4052
	Vulvar + Bradykinin	264	5472

Figure 2. Vulvar and vestibular fibroblasts from cases and controls express bradykinin receptor protein on their cell surface.

Flow cytometry was used to assess the relative surface expression of the BDKRB1 (panels A and C) and BDKRB2 (panels B and D) receptors on normal control fibroblasts (panels A and B) and LPV case fibroblasts (panels C and D). Expression in paired vestibular and external vulvar cells was examined with and without 1 μ M bradykinin treatment. Panel E lists the mean fluorescence intensities (MFIs) for all conditions, while panels A-D depict the expression plots. For both receptors, the shift in expression over the unstained control is generally greater in LPV case fibroblasts than in normal control fibroblasts, while the MFI is

slightly higher in vestibular versus vulvar cells and increases with bradykinin treatment. Therefore, both receptors are present in all strains tested, although BDKRB1 and BDKRB2 appear to be more highly abundant in LPV cases and in pain-associated vestibular fibroblasts.

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Figure 3. Total BDKRB1 and BDKRB2 protein is more abundant in cases versus controls. Western blotting with antibodies specific for BDKRB1 and BDKRB2 was performed to assess relative expression in total protein lysates (panel A). β-tubulin served as a control for normalization. Three paired case and three paired control fibroblast strains were examined. Overall, expression was markedly higher in cases versus controls, which is reflected in the densitometry plots in panel B. Furthermore, BDKRB2 was not only more abundant in cases, but it was also more abundant in vestibular versus external vulvar fibroblasts, in both cases and controls. A single asterisk denotes a significant difference between cases and controls, while two daggers indicate a significant between paired vestibular and vulvar strains ($n = 3$, $p > 0.05$)

Figure 4. Both case and control fibroblast strains respond to bradykinin in a dose-dependent manner.

The amount of IL-6 produced (as a measure of proinflammatory signaling) in response to challenge with bradykinin over a dose range of 0.001–10 μ M was measured. We assessed the response in paired control (panels A and B) and LPV case (panels C and D) strains. We found that bradykinin significantly induces IL-6 production (over vehicle control) at doses of 0.01 μ M and above and that the strength of the response is dose dependent. Furthermore, cases respond more strongly to bradykinin, producing ~2-fold higher amounts of IL-6 (versus controls). Vestibular fibroblasts also appear to produce more IL-6 than their external vulvar counterparts on average, but this finding is not statistically significant. A single asterisk denotes a significant difference between vehicle and treatment for either vestibular (light bars) or external vulvar (dark bars) cells ($n = 4$, $p < 0.05$).

Figure 5. Post-bradykinin stimulated fibroblast IL-6 production predicts mechanical pain threshold.

A multiple linear regression analysis was performed including two independent variables: presence of disease and the post-bradykinin stimulated IL-6 production, and one dependent variable: mechanical pain threshold following natural log transformation. This model was developed to ascertain whether post-bradykinin IL-6 production by location specific fibroblasts could predict pain level. Heteroskedasticity and collinearity were ruled out by inspection of residuals, Cook-Weisberg test, and pairwise correlation. The scatterplot, estimated regression of the model and 95% confidence intervals is graphically presented above.

Figure 6. Inhibiting the expression of BDKRB1 and BDKRB2 significantly reduces the amount of IL-6 produced by vestibular fibroblasts.

We used siRNAs against BDKRB1 and BDKRB2 to assess the role of these receptors in IL-6 production. We first confirmed successful knockdown of BDKRB1 and BDKRB2 using Western blotting (panel A). Band intensity, normalized to β-tubulin, was reduced by greater than 90% compared to control siRNA-treated cells. We then assessed the effect of siRNA treatment with either B1 or B2 alone or with both siRNAs together (B1+B2). Results are depicted for one normal control (panel B) and one LPV case (panel C). We found that treatment with either siRNA alone or in combination significantly reduced IL-6 production

in vestibular fibroblasts (light bars) compared to treatment with the control siRNA. For normal control fibroblasts, there were no significant differences in IL-6 production between single siRNA or combined siRNA treatment. In the cases, IL-6 production was lower in vestibular fibroblasts treated with both siRNAs versus either alone. No significant differences were noted between control and BDK siRNA-treated external vulvar cells (dark bars). However, IL-6 production in these cells was not significantly different from background (data not shown). Overall, inhibiting one or both BDK receptors significantly reduces IL-6 production in vestibular fibroblasts. A single asterisk denotes a significant difference between control and BDK siRNAs ($n = 4$, $p < 0.05$).

Figure 7. Chemical inhibition of BDKRB1 and BDKRB2 also reduces IL-6 production.

We used commercially available selective chemical inhibitors of BDKRB1 (R715) and BDKRB2 (HOE-140) to assess the impact of receptor inhibition using a second inhibitory method. We found that treatment with either R715 (panel A) or HOE-140 (panel B) significantly reduced the amount of IL-6 produced by vestibular fibroblasts treated with 0.1 μ M bradykinin following a 1 h pre-treatment with bradykinin receptor inhibitor. However, receptor inhibition did not affect IL-6 production by vulvar cells, which was maintained a relatively low level. A single asterisk denotes a significant difference between receptor inhibitor-treated cells and untreated cells ($n = 4$, $p < 0.05$).

Figure 8. Bradykinin turns on NF κ B expression, while inhibition of NF κ B abrogates IL-6 production.

We used an NF κ B reporter construct that produces luciferase when NF κ B-mediated transcription is activated to assess the ability of bradykinin to turn on the NF κ B signaling pathway in one set of LPV case and control fibroblast strains (panel A). All values are normalized to a constitutive renilla expressing plasmid, which adjusts for the number of viable cells in each well. The fold activity (y-axis) is plotted as treatment over vehicle. All values obtained were greater than one, which denotes activation over vehicle. Activity values for all fibroblast strains and treatments were significantly higher than the values obtained for vehicle ($n = 8$, $p < 0.05$), indicating that $0.1 \mu\text{M}$ bradykinin treatment turns on NF κ B in all fibroblast strains tested. We also compared the magnitude of induction by bradykinin to a known strong inducer of NF κ B signaling, IL-1 β (panel B). We found that activation with bradykinin was comparable to activation with IL-1 β , as there were no significant differences in the fold increase in NF κ B activity between bradykinin and IL-1 β stimulated cells ($n = 3$,

$P > 0.05$). We then evaluated the ability of BAY-11-7082, a widely used and commercially available NF κ B inhibitor, to reduce IL-6 production in two sets of LPV case strains challenged with 0.1 μ M bradykinin (panels C and D). We found that simultaneous treatment with BAY-11-7082 restored IL-6 release to background levels, which represents a significant reduction in IL-6 production versus cells challenged with bradykinin alone. A single asterisks denotes a significant difference between bradykinin-treated and bradykinin + BAY-11-7082-treated cells ($n = 4$, $p < 0.05$).

Table 1.

Gene names are listed with their descriptive functional category at the far left. Relative vestibular and external vulvar expression values are provided to the right of each gene name. “Not detected” denotes genes for which expression was not detected via RNAseq. Values are presented as the number of fragments per kilobase of exon per million fragments sequenced (FPKM).

Description	Gene	Vestibular Expression	Vulvar Expression
bradykinin (inducible) eceptor B1	BDKRB1	15.811	0.326
bradykinin (constitutive) receptor B2	BDKRB2	21.294	2.703
plasma kallikrein	KLKB1	0.001	0.006
pseudogene	KLKP1	not detected	not detected
HMW (high molec. wt.) precursor	KNG1	not detected	not detected
	KLK1	0.004	not detected
	KLK2	0.005	0.007
	KLK3	0.006	not detected
	KLK4	not detected	not detected
	KLK5	not detected	not detected
	KLK6	not detected	not detected
	KLK7	0.002	0.008
	KLK8	0.012	not detected
	KLK9	0.002	not detected
	KLK10	0.001	not detected
	KLK11	0.003	not detected
	KLK12	not detected	not detected
	KLK13	not detected	not detected
	KLK14	not detected	not detected
	KLK15	not detected	not detected