Relationship between bradykinin-induced relaxation and endogenous epoxyeicosanoid synthesis in human bronchi

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Eicosanoids are produced by cyclooxygenase (COX) and lipoxygenase (LOX) pathways that convert arachidonic acid (AA) into prostaglandins, leukotrienes, or hydroxyeicosatetraenonic acids (HETES), respectively (1, 10, 24, 38). Other eicosanoids, however, conversely display beneficial effects. For instance, epoxyeicosatrienoic acids (EET), produced by cytochrome P-450 (CYP450) epoxygenase, and 20-hydroxyeicosatetraenoic acid, produced by CYP450 ω-hydroxylase, have been described as anti-inflammatory and bronchorelaxing agents (7, 23, 33).

EET regioisomers, which are AA epoxide metabolites, are produced in epithelial and endothelial cells by epoxygenases, mainly by CYP450 2J2 and 2C9 isoforms (15, 37). They are metabolized in ASM cells and other cell types by the soluble epoxide hydrolase (sEH) into dihydroxyeicosatetraenoic acids (DHT), which are inactive metabolites (6, 8, 39). Four different EET regioisomers, namely 5,6-, 8,9-, 11,12-, and 14,15-EET, depending on the position of the epoxy group on the AA molecule, are found in numerous organs such as the lung, heart, kidney, liver, uterine myometrium, and brain (14, 15, 39). Of note, 14,15-EET is the most abundant regioisomer in lung tissues and one of the most active from a functional standpoint (39).

EETs have been proposed as potential endothelial- and epithelial-derived hyperpolarizing factors (EDHF and EpDHF, respectively) because of their effects on membrane potential, mainly on K+ currents (1, 4, 9). Their relaxing and hyperpolarizing effects are partially inhibited by 10 nM iberiotoxin (a large-conductance Ca2+-sensitive K+ (BKCa) channel blocker), by 27% following addition of 3 μM 14,15-epoxyeicosapentaenoic acid (5-Z)-enoic acid (an EET antagonist), and by 32% with 3 μM N-methanesulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH, an epoxyenase inhibitor). Hence, BK and 14,15-EET display net hyperpolarizing effects on smooth muscle cells that are related to the activation of BKCa channels and ultimately yielding to relaxation.

Data also indicate that 3 μM MS-PPOH reduced the hyperpolarizing effects of BK by 43%. Together, the present data support the current hypothesis suggesting a direct relationship between BK and the production of EET regioisomers. Because of its potent anti-inflammatory and relaxing properties, epoxyeicosanoid signaling may represent a promising target in asthma and chronic obstructive pulmonary disease.

The relaxing effects of these compounds on ASM cells are mediated through their hyperpolarizing effects and the modulation of contractile regulatory proteins (23). EET treatments on human bronchi have been demonstrated to modulate the activity of the 17-kDa myosin phosphatase inhibitor protein CPI-17 (23, 24). Following G protein-coupled receptor (GPCR) activation, the subsequent rise in intracellular Ca2+ leads to the formation of calcium/calcmodulin complexes that activate myosin light chain kinase. This activation in turn phosphorylates the 20-kDa myosin light chain, resulting in actin-myosin interactions and contraction (32). GPCR activation can also induce protein kinase C-dependent CPI-17 phosphorylation, which leads to phosphorylation of the myosin phosphatase targeting protein myosin-binding subunit of myosin light chain phosphatase-1 and resulting in the inhibition of myosin light chain phosphatase, hence facilitating the maintenance of contractile tone (30, 32).

EETs have also been shown to display anti-inflammatory and anti-hyperreactive effects on human bronchi through specific activation of the peroxisome proliferator-activated receptor-γ (PPARγ) (18, 23, 25). Using an hyperreactive model of...
human bronchi induced by 10 ng/ml tumor necrosis factor (TNF-α) treatments and tension measurements, our group demonstrated that 300 nM 14,15-EET significantly reduced TNF-α-triggered hyperreactivity (23). Activation of the PPARγ receptor also induces an upregulation of inhibitory factor κBα (IkBα), which inhibits the translocation of NF-κB into the nucleus (26, 34). Modulation of the NF-κB pathway decreases the expression of different proinflammatory cytokines and proteins, such as IL-2, IL-6, and cyclooxygenase 2 (26, 34).

Over the past decade, several mechanisms of action of EETs have been defined (1, 23, 24). However, it is still unknown which stimulus triggers their endogenous production in the human lung. Our working hypothesis stipulates the existence of a putative relationship between the activation of bradykinin (BK) receptors and the endogenous production of EET. Indeed, EETs are suspected to be EpDH molecules, although many groups have demonstrated that epithelial BK receptor activation induces the production of other EpDHFs, such as nitric oxide (NO) and prostacyclin (PGI2) (4, 19, 27).

BK, a member of the kinin family, is a vasoactive nonapeptide produced in plasma by kallikrein, a subgroup of serine protease enzymes, from kininogen (produced in the liver) and is mainly metabolized in the lung by metalloproteases such as neutral endopeptidase (or nephrin) and angiotensin-convert- ing enzyme (19, 22, 28). BK is primarily produced in response to inflammation, infection, or tissue injury (3, 12), and it is known to induce beneficial antihypertensive, antiproliferative, and antifibrogenic effects (21, 22). However, adverse effects related to this peptide have also been reported, such as an increase in vascular permeability by activating endothelial cells, which contribute to inflammatory processes and angiogenesis (3, 12). Its physiological effects are produced through the activation of two different GPCR receptors, namely B1R and B2R (5, 20, 21). The latter, B2R, which is constitutively expressed in many cell types, displays a higher affinity to BK than B1R (19, 20, 28). Conversely, B1R is an inducible receptor in response to tissue damage and inflammation. It is preferentially activated by des-Arg9-BK (5, 20). Both of their signaling pathways are related to the pertussis toxin-insensitive Gq-type protein, which activates phospholipase C-β that in turn produces inositol 1,4,5-triphosphate (IP3) and leads to intracellular Ca²⁺ release mainly through IP3 receptors (3, 19, 22). This mode of action on epithelial and endothelial cells stimulates the endogenous production of NO and PGL2, which, respectively, trigger an increase in intracellular cGMP and cAMP, both resulting in smooth muscle relaxation (20, 22).

The aim of the present study was to assess whether there is a relationship between BK stimulation and the endogenous production of EET in human bronchi using a well-established in vitro model of cultured tissues (23) in which several mechanical and electrophysiological measurements were performed. Herein, we report the first evidence of a relationship between BK effects and endogenous EET synthesis in human bronchi.

**MATERIALS AND METHODS**

**Study population and lung resection samples.** This study was approved by the institutional Ethics Committee of the Centre Hospitalier Universitaire de Sherbrooke (protocol no. 05 088 S2-M2) and was designed in collaboration with the Service of Thoracic Surgery and the Department of Pathology. The experiments were primarily performed on healthy human lung tissues obtained from patients undergoing lobectomy or pneumonectomy for adenocarcinoma resection. All lung tissues used were from areas distant from the tumor site and devoid of any carcinoma cells as ascertained by the pathologist. Lungs from patients with hazardous risk of contamination, such as tuberculosis or methicillin-resistant *Staphylococcus aureus*, were excluded from the study by the pathologists.

**Tissue preparation and culture.** Lung specimens were placed in a sterile saline solution and transported from the operating room to the pathology department. Following macroscopic analysis, a slice of the resection was provided by the pathologist, distant from the carcinoma, such as to ensure that there were no metastatic nodules. The resection was transported to the laboratory in a Krebs solution containing (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.1 dextrose, at pH 7.4. Bronchi were thereafter dissected under binocular control in a level-2 culture room and transferred in 24-well culture plates containing Dulbecco’s modified Eagle’s (DMEM)-F12 culture medium (1 ml/well) supplemented with 1% penicillin (10 U/ml) and streptomycin (100 µg/ml). All culture plates were maintained in a humidified incubator at 37°C under 5% CO₂ for a maximum culture time of 18 h to minimize epithelial damage and loss of reactivity until mechanical measurements were performed.

**Mechanical tension measurements.** Mechanical tension measurements were performed using an isolated organ bath system (Radnotti Glass Technology, Monrovia, CA) as previously described (24). Bronchial rings were mounted in baths containing 6 ml Krebs solution maintained at 37°C and continuously gassed with carbogen (95% O₂-5% CO₂). A basal tension of 0.8 g was applied to each bronchi followed by an equilibration period of 30 min with three washouts every 10 min. Passive and active tensions were assessed using FT03 Grass transducer systems coupled to Polyview software (Grass-Astro-Med, West Warwick, RI) to allow data acquisition and analysis.

**Microelectrode measurements.** The transmembrane potential measurements were assessed using a microelectrode setup as previously described (4, 24). Bronchi were longitudinally cut and fixed in the middle chamber of a three-compartment system (3 ml capacity) with the luminal side and ASM cells facing upward. Experiments were performed in a standard Krebs solution, maintained at 37°C, and gassed with carbogen (95% O₂-5% CO₂). A basal tension of 0.8 g was applied to each bronchi followed by an equilibration period of 30 min with three washouts every 10 min. Passive and active tensions were assessed using FT03 Grass transducer systems coupled to Polyview software (Grass-Astro-Med, West Warwick, RI) to allow data acquisition and analysis.

**Transepithelial electrical resistance measurements and cell culture.** Transepithelial electrical resistance (TEER) measurements were performed on A549 cells (Clonetics, San Diego, CA) grown at the air-liquid interface in Transwell plates (Corning, NY) as previously described (11). The cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Burlington, Canada) at pH 7.4, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO, Burlington, Canada) at 37°C in 5% CO₂. TEER was measured between the apical and the basolateral compartment following the addition of 500 μl RPMI medium in the apical chamber with low-resistance chopstick electrodes and an EVOM ohmmeter (World Precision Instruments).

**Drugs and chemical reagents.** 14,15-EET, 14,15-epoxycis-5(Z)-enoic acid (14,15-EEZE), IbTx, and U-46619 were purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in 100% ethanol. Lipids were stored at stock concentrations of 312, 308, and 936 μM. Distilled water was used for 0.5 μM IbTx. Methacholine chloride (MCh) was purchased from Sigma (St. Louis, MO), dissolved in distilled water, and stored as a 100 mM solution. DMEM-F12, A549, H9260, H9251, B, B(1), B(2), receptor, B2R, B1R, AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00379.2012 • www.ajplung.org
BSA, and penicillin-streptomycin were purchased from GIBCO Invitrogen. RPMI medium was purchased from Invitrogen. N-methanesulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH) was a gift from the laboratory of Dr. John Falck (Southwestern Medical Center, Dallas, TX), dissolved in 100% ethanol, and stored as 10 μM stock solution. BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) powder was a gift from Dr. Fernand Gobeil, Jr. (Université de Sherbrooke), dissolved in distilled water, and stored as 10 mM stock solution.

Data analysis and statistics. Results are expressed as means ± SE, with n indicating the number of experiments. Statistical analyses were performed with the Wilcoxon Signed-Rank test or one-way ANOVA. Differences were considered significant when P < 0.05. All statistical analyses were performed with Sigma Plot 12.0 (SPSS-Science, Chicago, IL).

RESULTS

Sample population. Among the 64 patients from which written informed consent was obtained for the collection of a lung resection sample following lobectomy or pneumonectomy, 43 specimens (67%) were suitable for mechanical and electrophysiological experiments and were thus included in this study. Among the 43 resection samples processed, 68% were diagnosed for adenocarcinoma, whereas 21% were for epidermoid cancer. The remaining 11% were for non-small-cell carcinoma, mucoepidermoid cancer, and a single colon cancer metastasis. In all cases, specimens retrieved for the experiments performed herein were taken well distant from the tumor, as reported previously (23).

Relaxing effects of BK and 14,15-EET on human bronchi. Fresh human bronchi were mounted in an isolated organ bath system for isometric tension measurements to assess their physiological reactivity and pharmacological properties. Bronchi were first challenged with 1 mM MCh to test ASM reactivity. Bronchi were then precontracted with 30 nM of the TP-receptor agonist U-46619 before addition of either 1 mM BK or 1 mM 14,15-EET (Fig. 1A), both latter conditions inducing significant transient relaxations. Figure 1B shows a bar histogram of the mean tensions induced by 30 nM U-46619 with a mean value of 0.35 ± 0.07 g for the first challenge and 0.39 ± 0.07 g for the second challenge, along with the residual tension, following the addition of 1 mM BK (0.17 ± 0.03 g) and 1 mM 14,15-EET (0.18 ± 0.02 g). Figure 1C quantifies the mean relative relaxing effects of BK and 14,15-EET, which resulted in inhibitory effects of 44.8 ± 2.8 and 41.7 ± 6.4%, respectively, on the tension developed by U-46619. Thus, at the concentration used, both molecules induced similar relaxing effects. Of note, 1 mM BK had no relaxing effect after epithelial cell removal (data not shown).

Concentration-dependent relaxing effects of BK and 14,15-EET. Isometric tension measurements were also performed to determine the effect of cumulative concentrations of 14,15-EET on precontracted human bronchi. A concentration of 30 nM U-46619 was consistently used to stimulate the bronchial rings, and, upon

Fig. 1. Relaxing effect of 14,15-epoxyeicosanoids (14,15-EET) and bradykinin (BK) on human bronchi precontracted with U-46619. A: representative recordings of BK and 14,15-EET relaxation on human bronchi precontracted with 30 nM U-46619. B: bar histogram displaying the contractile effect of 30 nM U-46619 and residual tension following addition of 1 μM 14,15-EET and 1 μM BK. C: quantification of 14,15-EET and BK relaxations as a percentage of the tension triggered by 30 nM U-46619. *Significant difference with P < 0.05.
reaching a plateau, cumulative concentrations of 14,15-EET (from 0.01 to 3 µM) were sequentially added, resulting in concentration-dependent relaxations (Fig. 2A). A similar set of experiments was also performed with cumulative concentrations of BK (Fig. 2B).

Figure 2C shows the cumulative concentration response curve to BK and 14,15-EET. Curve fittings allowed calculation of an apparent IC50 value of 0.28 ± 0.01 and 0.27 ± 0.01 µM, respectively, for BK and 14,15-EET on pharmacologically induced tone. Of note were the striking similarities of IC50 values between exogenous BK and 14,15-EET.

Effect of various inhibitors on U-46619 and BK mechanical responses. The effects of various inhibitors interacting with the EET signaling pathway were assessed on both U-46619-induced tension and BK-relaxing effects. Figure 3A shows rep-

Fig. 2. Effect of cumulative concentration-response curves (CCRC) to BK and 14,15-EET on human bronchi. A: representative trace of the concentration-dependent relaxing effect of 14,15-EET. B: representative trace of BK on a human bronchus precontracted with 30 nM U-46619. C: quantification of CCRC to BK and 14,15-EET allowed determination of IC50 values of 0.28 and 0.27 µM, respectively, on the tension developed upon thromboxane-prostanoid receptor stimulation.

Fig. 3. Effects of various inhibitors on the relaxing effect of BK on human bronchi precontracted with U-46619. A: representative trace of the BK-relaxing effect in the absence or presence of the epoxygenase inhibitor 3 µM 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE). B: bar histogram showing the effect of pretreatment with 10 nM iberiotoxin (IbTx, a specific BKCa blocker), 3 µM MS-PPOH on the tension induced by 30 nM U-46619 on human bronchi. C: quantification of the BK-relaxing effect in the absence or presence of these inhibitors. *Significant difference with P < 0.05.
representative recordings of BK-relaxing effects on precontracted human bronchi, in the absence (control, Fig. 3A, left) and the presence of 3 µM MS-PPOH, a CYP450 epoxygenase inhibitor (Fig. 3A, right). Pretreatment with MS-PPOH reduced the relaxing tension induced by 1 µM BK. In these sets of experiments, each bronchus served as its own control. Quantitative analysis revealed that, in control conditions, 30 nM U-46619 induced an average tension of 0.28 ± 0.04 g (Fig. 3B), whereas, following pretreatment with 10 nM IbTx, a specific BKCa blocker, the tonic response to 30 nM U-46619 was significantly increased by 42.1% compared with control. Pretreatment with 3 µM 14,15-EEZE, an antagonist of EET effects (24), had no effect on the tension induced by 30 nM U-46619, whereas 3 µM MS-PPOH pretreatment induced a mean increase of 42.7% compared with control (Fig. 3B). Under control conditions, the relaxing effect generated by BK represented 43.6 ± 2.6% of the tension developed by 30 nM U-46619 (Fig. 3C). Moreover, the mean relaxing effects of BK on 10 nM IbTx-, 3 µM 14,15-EEZE-, and 3 µM MS-PPOH-pretreated bronchi were 25.0 ± 2.8, 31.9 ± 4.7, and 29.5 ± 3.2%, respectively. Last, inhibitors displayed a marked reduction in BK-relaxing effects of 42.6, 26.9, and 32.3%, respectively (Fig. 3C).

Effect of an epoxygenase inhibitor on the hyperpolarizing effect of BK. Microelectrode experiments were performed to assess the effect of the CYP450 epoxygenase inhibitor MS-PPOH on the hyperpolarizing effect of BK on ASM cells. Figure 4A shows a functional diagram of the microelectrode recording system. The right compartment corresponds to the degassing chamber of the perfusion system; the middle chamber, corresponding to the recording compartment, was perfused by a constant laminar flow (3 ml/min) while the left compartment contained the physiological solution for which the level was maintained by low suction. Figure 4B displays representative traces of the hyperpolarizing effect of BK in the absence (control) or presence of 3 µM MS-PPOH, whereas mean responses are quantified in a bar histogram (Fig. 4C). The average membrane potential under control conditions (without treatment) was −46.9 ± 1.2 mV, whereas the addition of 3 µM BK induced a 16.1 ± 1.1-mV hyperpolarization, which led to an average membrane potential of −63.0 ± 2.2 mV. When the bronchi were pretreated for 15 min with 3 µM MS-PPOH, the hyperpolarizing effect of BK (−9.2 ± 0.5 mV) was reduced by 42.9% (Fig. 4C). All recordings were validated at the end of each measurement upon removal of the microelectrode from the tissue and consisted of a mandatory return of the reference.
potential to a value of $0 \pm 2$ mV. Note that 3 µM BK had no hyperpolarizing effect upon removal of epithelial cells (data not shown).

**Inhibition of endogenous EET synthesis on BK-induced transepithelial resistance.** TEER measurements were performed to assess the effect of CYP450 epoxygenase inhibitor pretreatment on the effect of BK on transepithelial resistance. Figure 5A shows the functional diagram of the TEER system with the Transwell plates in which epithelial A549 cells were grown and the low resistances connected to the ohmmeter. Compared with the control value ($157.8 \pm 3.2$ Ω), 3 µM BK induced a significant reduction in transepithelial resistance as a function of time with a maximum reduction of $20.1 \pm 1.4$ Ω after 3 min (Fig. 5B). Following a 15-min pretreatment with 3 µM MS-PPOH, 3 µM BK induced a maximal reduction in transepithelial resistance of $-9.9 \pm 0.5$ Ω corresponding to a 50.7% reduction in BK response (Fig. 5B).

**DISCUSSION**

In the present study, we assessed the putative relationship between the mode of action of BK and endogenous EET production on the functional properties of human bronchi. The most relevant finding of this work is that a reduction in EET bioavailability significantly reduced the effects of BK on both tone and electrophysiological parameters. For the first time, we report that both BK and 14,15-EET trigger relaxing effects of similar amplitude, time course, and IC$_{50}$. Furthermore, we demonstrate that treatments with inhibitors that interfere with the signaling pathway of EET, such as a BK$_{c_a}$ channel blocker and a CYP450 epoxygenase inhibitor, increase TP receptor-induced tension and reduce BK-stimulated relaxation in human bronchi. These findings strongly suggest a possible relationship between BK stimulation and endogenous EET production in human epithelial and ASM cells.

**Comparative effects of BK and 14,15-EET.** It has been suggested that EETs could represent a valuable therapeutic target because of their beneficial effects on smooth muscle cells (13, 33) as well as for their anti-inflammatory actions on coronary artery and human bronchi (7, 25). To develop this specific approach, it was of prime interest to first establish which ligand and endogenous mechanisms trigger EET regiosomer synthesis. Because of their hyperpolarizing effects on membrane potential, EETs have been proposed as EpDHF (4, 9), whereas BK is well known to induce the production of other EpDHF candidates (19, 27).

In this study, we first compared the relaxing effects of both compounds. Both BK and exogenous 14,15-EET relaxed U-46619-precontracted human bronchi. On the one hand, the transient relaxing effects of EET are likely related to a rapid hydrolysis of the EET epoxide group by sEH, which in turn produces the biologically inactive DHET (6). As for the relaxing effect of BK, its action could be explained by either an inactivation of BK receptors, the transient production of relaxing compounds (NO, PGI$_2$), or by the degradation of EET. Results further revealed that both BK and EETs induced the same relaxing time course on precontracted human bronchi, hence providing the first clue as to the putative relationship between these relaxing agents. Given the lack of significant difference between the relaxing effects of both molecules (44.8 ± 2.8% for 1 µM BK and 41.7 ± 6.4% for 1 µM 14,15-EET), we proceeded to further pursue their potential relationship. Hence, the effect of the combined addition of both compounds (1 µM) on U-46619-precontracted human bronchi was assessed, with no observable difference found between the relaxing effects of both molecules, alone or in combination (44.2 ± 4.3%).

**Inhibitors and blockers modify the mechanical responses of U-46619 and BK.** There are several inhibitors that can be used to interact with the AA metabolic pathway or with its effectors (such as membrane ionic channels or epoxyeicosanoid antagonists). Indeed, our data demonstrate that the mean tonic responses to the TP receptor agonist U-46619 were significantly increased when BK$_{c_a}$ channels were blocked with 10 nM IbTx. The use of IbTx decreases membrane K$^+$ conductance, which likely facilitates smooth muscle cell depolarization, hence leading to increased tension. This ion blocker was also found to reduce the relaxing effect of BK by 42.6%. This result is in agreement with a previous observation by Liu et al. (17) on cultured smooth muscle cells, thus indicating that both
BK and EETs act through the activation of this large-conducting $K^+$ channel.

In contrast to our expectations, a 10-min pretreatment with the EET antagonist 14,15-EEZE had no significant effect on U-46619-induced tension. It was anticipated that the inhibition of the effect of EET by the antagonist would increase TP receptor-induced tension because of the potential opposing effects between these lipid mediators (31). On the other hand, 14,15-EEZE pretreatment did have a significant inhibitory effect on BK-induced relaxation.

It was previously shown that a 15-min pretreatment with 3 $\mu$M MS-PPOH (a CYP450 epoxygenase inhibitor that minimizes the endogenous production of EETs) (23, 24) significantly increased U-46619-induced tension in human bronchi (31). We now demonstrate that CYP450 epoxygenase inhibition, which leads to a decrease in EET bioavailability, significantly reduced BK-induced relaxation (as reported in Fig. 3C).

Altogether, these data strongly suggest a relationship between the effects of BK and the endogenous production of EET on functional properties in human bronchi. However, MS-PPOH only partially inhibited the relaxing effects of BK (32.3%). The residual effect observed is likely because of synthesis of other EpDHFs stimulated by BK. Indeed, as we previously reported, BK induces NO and PG12 synthesis. In the presence of MS-PPOH, these molecules are produced following BK stimulation and maintain their physiological effects, which consist of cell membrane hyperpolarization leading to smooth muscle cell relaxation.

Hyperpolarization by BK is partially reversed by CYP450 epoxygenase inhibitor. It has been shown that BK activates BKCa channels and hyperpolarizes ASM cells (17). We therefore performed microelectrode measurements to assess whether the endogenous production of EET may be involved in this effect. Our data demonstrate that blocking EET production with the epoxygenase inhibitor MS-PPOH significantly reduced the hyperpolarization of ASM cells by BK. These results indicate that the effects of BK on smooth muscle cells are partially achieved through the synthesis of AA-derived epoxide metabolites. We suspect that this effect on membrane potential may be related to the activation of BK receptors on epithelial cells, since epithelial layer removal abolished the electrophysiological and mechanical responses, hence justifying the pursuit of experiments on human epithelial cells.

The effect of BK on transepithelial resistance is modulated by MS-PPOH. The data presented herein demonstrate a relationship between the effects of BK and the synthesis of EET in the functional activities of bronchi and in ASM electrophysiological properties. As reported previously, bronchial EET synthesis occurs in epithelial cells, which express CYP450 epoxygenase (15). Moreover, it is well established that activation of BK receptors on epithelial cells leads to EpDHF release (19). In a complementary set of experiments, we attempted to demonstrate that the relationship previously observed between BK and EETs also exists in epithelial cells. To confirm this working hypothesis, TEER measurements were performed on epithelial cells treated with BK in the absence or presence of the CYP epoxygenase inhibitor MS-PPOH. These experiments were performed on A549 cells, which are adenocarcinoma-derived human alveolar basal epithelial cells. In the absence of BK treatment, the mean transepithelial resistance was fairly low at 157.8 $\Omega$. A 15-min treatment with MS-PPOH alone induced a slight nonsignificant increase (160.8 $\Omega$). However, upon addition of 3 $\mu$M BK, a slow decrease in resistance was recorded that was significant after 1 min until the 4th min, followed by a rapid recovery to control values after 5 min. Such rapid recovery kinetics suggests the presence of an efficient biochemical process that could modify either the effects of BK and/or EET signaling and in which the role of SEH could be suspected.

Together these data confirm that BK stimulation of airway epithelial cells likely induces endogenous EET synthesis. One mechanism to explain this observation would be that activation of BK receptors on epithelial cells induces an increase in intracellular Ca$^{2+}$ (28). Indeed, it has been shown that the activation of specific PLA2 isoforms is Ca$^{2+}$-dependent (2). This PLA2 activation leads to an increase in AA substrate availability to be metabolized by CYP450 epoxyenases into EET. However, AA can be metabolized into various compounds. Our current data are consistent with our working hypothesis stating that activation by BK directly (or indirectly) stimulates an increase in epoxyenase activity, which leads to EET synthesis and enhanced relaxation. Further investigations, such as liquid chromatography or gas chromatography-tandem mass spectrometry-based measurements, are warranted to further establish this specific aspect.

In summary, despite 30 years of intensive research to fully understand the mode of action of BK, certain effectors remain to be identified. Whereas our previous studies enabled us to uncover the implication and roles of EET regioisomers in the bronchial wall (23, 24), we now demonstrate an increased bioavailability of EET in lung tissues in response to BK. Our current data also suggest that EETs display potent paracrine and autocrine biological activities in vitro. Such data from this emerging field of research may provide alternative options for the prevention and treatment of asthma and chronic obstructive pulmonary disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.T. and E. Rousseau conception and design of research; Y.T. performed experiments; Y.T. and E. Rizcallah analyzed data; Y.T. and E. Rousseau interpreted results of experiments; Y.T. prepared figures; Y.T. drafted manuscript; Y.T., M.S., C.S., E. Rizcallah, and E. Rousseau edited and revised manuscript; Y.T., M.S., C.S., E. Rizcallah, and E. Rousseau approved final version of manuscript.
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