Functional effects of 20-HETE on human bronchi: hyperpolarization and relaxation due to BK$_{	ext{Ca}}$ channel activation

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Submitted 12 April 2007; accepted in final form 24 July 2007

The physiological roles and modes of action of 20-hydroxyeicosatetraenoic acid (20-HETE) have been studied in various tissues (25, 27, 29), including rodent lungs (4); however, its putative role in human airway smooth muscle (ASM) cells has yet to be well defined. 20-HETE is a bioactive eicosanoid synthesized from free arachidonic acid (AA) by cytochrome P-450 (CYP-450) ω-hydroxylase. 20-HETE is an important modulator of vascular, kidney, gastrointestinal, and bronchial cell reactivity (4, 25, 30). The CYP-450 enzymes are predominantly detected in the liver (23), heart (29), vasculature, gastrointestinal tract, kidney (27), and lung (31). Moreover, its production may be affected in vivo under certain pathological conditions (16). In guinea pig, 20-HETE induces an increase in ASM basal tone (4), an effect largely due to specific electrophysiological processes such as direct activation of various surface membrane ionic conductances. These include nonselective cationic channels such as type 6 canonical transient receptor potential (4, 26). In contrast, 20-HETE relaxes rabbit bronchi preconstricted with histamine (12). These relaxant effects are inhibited by indomethacin, a cyclooxygenase inhibitor, or by removal of the endothelial lining (12).

It has also been reported that 20-HETE modulates vascular tone and cell proliferation and appears to play a role in the regulation of blood pressure and myogenic responses (25). Several different intracellular signaling pathways are known to mediate these biological responses to 20-HETE. For example, 20-HETE can activate protein kinase C, mitogen-activated protein kinase, Rho-kinase, and cytosolic phospholipase A2 (20, 21, 24). A major metabolite of 20-HETE is 20-carboxy-arachidonic acid (20-COOH-AA), which has been shown to dilate porcine coronary microvessels and inhibit ion transport in kidney tubular cells (5, 6, 13). One of the challenging issues in this field is that there are presently no specifically identified or cloned 20-HETE receptors.

The aim of this study was to assess the physiological effects of 20-HETE on ASM at the tissue, cellular, and molecular levels using an organ culture model derived from human bronchi. Complementary approaches were used to perform: 1) tension measurements on human bronchial rings, 2) membrane potential measurements using the classic microelectrode technique, and 3) analyses of the effects of 20-HETE on unitary BK$_{	ext{Ca}}$ channels reconstituted into planar lipid bilayers. Herein, we report the first evidence that 20-HETE induces concentration-dependent and iberiotoxin-sensitive relaxations, as well as hyperpolarizations of resting membrane potential in ASM derived from human bronchi. These effects are correlated with a direct activation of BK$_{	ext{Ca}}$ channels in ASM cells as shown by single-channel analysis.

MATERIALS AND METHODS

Isolation and organ culture of human bronchi. The study was approved by our local institutional ethics committee (protocol number CRC 05-088) and informed consents were given by human subjects. Human lung tissues were obtained from 27 patients undergoing surgery for lung carcinoma. Following lobectomy and transport in sterile physiological saline solution, lung samples, distant from the malignant infiltration, were dissected by the pathologist. The absence of tumoral infiltration was retrospectively established in all bronchi by pathological analysis. Tissue samples were immediately placed in Krebs solution (composition in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 25 NaHCO$_3$, and 11.1 glucose), previously bubbled with 95% O$_2$ and 5% CO$_2$, pH 7.4, at 22°C and then immediately transported to a level two-culture room. After removal of connective tissues and adhering parenchyma, paired rings of similar weight and length (inner diameter of 0.5–0.8 mm) were microdissected from the same bronchus segment. The bronchial
rings were placed in individual wells of 12-well culture plates containing DMEM-F-12 culture medium (2 ml/well) supplemented with 0.3% penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Culture plates were placed in a humidified incubator at 37°C under 5% CO₂. Explants were maintained in culture for 1–3 days (17, 19).

Isometric tension measurements. The mechanical effects induced by specific agonists and eicosanoids were measured as previously reported (18). Bronchial rings were mounted in isolated organ baths containing 6 ml of Krebs solution at 37°C, continually gassed with the 95% O₂–5% CO₂ mixture and to which an initial load of 0.6 g was applied. Tissues were allowed to equilibrate for 1 h in Krebs solution and were washed out every 15 min. Passive and active tensions were assessed using transducer systems (Radnoti Glass Tech., Monrovia, CA) coupled to Polyview software (Grass-Astro-Med, West Warwick, RI) for facilitating data acquisition and analysis.

Microelectrode measurements. A longitudinal section was performed to expose the luminal face of the bronchi. The strips were affixed with the ASM facing up, in the middle chamber (capacity 3 ml) of a tri-compartment system, in which temperature was maintained at 37°C as previously described (3, 19). The tissues were superfused at a constant flow rate of 2 ml/min with standard Krebs solution and allowed to equilibrate for 20 min. Membrane potential was measured using conventional intracellular borosilicate microelectrodes, filled with 3 M KCl with a resistance ranging from 30 to 50 MΩ. Measurements were performed with a KS-G-700 amplifier from World Precision Instruments (Sarosota, FL). Electrical signals were continuously monitored on a TDS 310 oscilloscope (Tektroniks, Beaverton, OR). The membrane potential was digitized and recorded using a Digidata 1200B interface and Axoscope 9.0 software from Axon Instruments (Union City, CA).

Preparation of bronchial microsomal fractions and channel reconstitution. Preparation of human crude ASM microsomal fractions and planar lipid bilayers (PLB) was carried out as described previously (1). Two chambers, denoted cisand trans, were separated by a septum with a 250-μm-diameter aperture. The aperture was pretreated with a mixture of phospholipids: phosphatidylethanolamine-phosphatidylserine-phosphatidylcholine at a ratio of 3:2:1 (in 25 mg/ml chloroform). This same mixture of phospholipids dissolved in decane was used to form the PLB. The membrane vesicles (10–60 μg of proteins) were added in the cis chamber, which was connected to the head stage of a voltage-clamp amplifier (model 8900; Dagan, Minneapolis, MN). To facilitate the fusion, the experimental chambers contained the following solutions: 250 mM KCl cis – 50 mM KCl trans plus 20 mM K-HEPES and 10 μM free Ca²⁺ (109 μM CaCl₂ + 100 μM K-EGTA), pH 7.4. BKCa channel activities were analyzed in terms of current amplitudes and channel open probability (Po) values with Clampfit 9 software (Axon instruments).

Drugs and chemical reagents. 20-HETE, 20-OH-PGE₂, and AA were obtained from Cayman Chemical (Ann Arbor, MI), dissolved in 100% ethanol (EtOH), and stored as 1 mM stock solutions. Iberiotoxin and indomethacin were purchased from Calbiochem (San Diego, CA and VWR, Montreal, Canada). Methacholine chloride (MCh) was purchased from Sigma (St. Louis, MO). DMEM-F-12 and penicillin-streptomycin were purchased from Gibco Invitrogen (Burlington, ON, Canada). N-methylsulfonfyl-12,12-dibromododec-11-enamide (DMS) was a kind gift from the laboratory of Dr. J. R. Falck, Univ. of Texas Southwestern Medical Center at Dallas (Dallas, TX).

Data analysis and statistics. Results are expressed as means ± SE with n indicating the number of experiments. Statistical analyses were performed using the Student’s t-test or by one-way ANOVA. Differences were considered significant when P < 0.05. Data curves fittings were performed using Sigma Plot 9.0 (SPSS-Science, Chicago, IL) to determine IC₅₀ values (18).

RESULTS

Effect of 20-HETE on human bronchial smooth muscle tension. Tension measurements were performed on human bronchial rings to test the effect of 20-HETE on ASM tone. Tissues were subjected to 0.8 g basal tone, and cumulative concentrations of 20-HETE (0.3–10 μM) resulted in concentration-dependent relaxing effects (Fig. 1A). 20-HETE (1 μM) yielded a mean relaxation of 1.2 ± 0.2 mN on human bronchi. Then, the effects of 20-HETE were assessed on the active tone generated by 1 μM MCh. Once the plateau phase of precontracted tissues was reached, cumulative concentrations of 20-HETE were added, resulting in concentration-dependent relaxing effects (Fig. 2A). The relaxing effects of 20-HETE were also assessed on AA-precontracted bronchi (data not shown). Figure 1C demonstrates the concentration-dependent relaxing effects induced by 20-HETE on 1 μM MCh and 1 μM AA-precontracted bronchi, with IC₅₀ values of 0.30 ± 0.03 and 0.35 ± 0.03 μM, respectively. Moreover, the effects of 20-HETE were assessed in the presence of 100 nM pertussis toxin.
(PTX), a $G_i$ protein inhibitor. The relaxing effects induced by 20-HETE on bronchial rings were not modified by PTX pretreatment (data not shown), suggesting that 20-HETE action does not involve the activation of a $G_i$ protein-mediated process in human ASM cells.

**Role of endogenous 20-HETE production in human bronchi.** To assess the role of the putative endogenous production of 20-HETE, DDMS, a specific CYP-450 $\omega$-hydroxylase inhibitor, was used (5). Thirty micromolars of DDMS had only a low biphasic effect on basal tone from human bronchi; however, it amplified the MCh responses as demonstrated in Fig. 2, A and B. Also, following DDMS pretreatment, the tonic responses to AA were increased by 46% (Fig. 2, C and D). Thus $\omega$-hydroxylase inhibition and lower endogenous production of 20-HETE facilitate MCh- and AA-induced tensions.

**Effect of cyclooxygenase inhibition on 20-HETE relaxing responses.** It was previously reported that in rabbit lung, 20-HETE responses were inhibited in the presence of indomethacin, a nonselective cyclooxygenase (COX) inhibitor (7, 12). Thus the relaxing effects of 20-HETE were assessed in the absence and the presence of 10 nM indomethacin (Fig. 3A). Indomethacin displayed well-characterized relaxing effects of low amplitude on the resting tone. However, quantitative analysis demonstrated that the relaxations induced by 20-HETE were not significantly modified by indomethacin pretreatment (Fig. 3, A and B). 20-OH-PGE$_2$, a putative 20-HETE metabolite, was also tested on 1 $\mu$M MCh-precontracted human bronchi (Fig. 3C). Data analysis is summarized in Fig. 3D. Three micromolars of 20-OH-PGE$_2$ induced a slight (7.5%) relaxation on MCh-precontracted bronchi. Together, these results indicate that the relaxing responses to 20-HETE are not related to an intracellular COX-derived prostanoid metabolite.

20-HETE relaxing effect is sensitive to IbTx. The relaxant effect of 20-HETE was assessed in the presence of 10 nM IbTx. Figure 4A depicts two sequential recordings in which IbTx pretreatment resulted in a significant inhibition of the amplitude of relaxation induced by 20-HETE compared with the control response. Quantitative analysis of the data is summarized in Fig. 4B. Preincubation with 10 nM IbTx yielded a 90% inhibitory effect on the relaxation induced by 1 $\mu$M 20-HETE, suggesting that activation of BKCa channels likely controls relaxations induced by this eicosanoid in human bronchi. These observations are consistent with previous data attesting to the activation of membrane conductances in various smooth muscle tissues (4). Note that in the presence of IbTx, the amplitude of the tonic responses to the muscarinic agonist was not modified.

20-HETE effect on ASM membrane potential. The effects of 20-HETE on ASM cell membrane potential were verified following microelectrode impalement on human bronchial explants. Figure 5A illustrates a representative recording of the hyperpolarizing effects induced by cumulative additions of 20-HETE (0.1–10 $\mu$M) from a resting membrane potential of −52 mV. At the end of each experiment, the microelectrode was removed from the ASM cell to validate the recordings. The mean electrophysiological effects of 20-HETE on ASM tissues are shown in Fig. 5B. Concentration-response curves were obtained, and a maximal hyperpolarizing effect of
−11.1 ± 0.8 mV was recorded upon addition of 10 μM 20-HETE. Following 10 nM IbTx pretreatments, the mean membrane potential value determined upon 1 μM 20-HETE addition was −53.4 ± 0.9 mV, which was not significantly different from the control value (−52.5 ± 1.0 mV).

**Effect of 20-HETE on activation of reconstituted BKCa channels in PLB.** The PLB technique allows one to test the direct putative effect of a specific compound with a single channel activity, in the absence of cellular regulations (1). To assess the effects of 20-HETE on channel activity, BKCa channels were reconstituted from ASM vesicles in an asymmetrical (50/250 mM) KCl buffer system, initially containing 10 μM free Ca²⁺ concentration ([Ca²⁺]). [Ca²⁺] was reduced upon addition of precalibrated concentrations of EGTA to the transchamber (cytoplasmic side of the channel) after which channel Pₒ was monitored (1). Pₒ values decreased as a function of [Ca²⁺], attesting to the Ca²⁺ sensitivity of the reconstituted channels. Figure 6A illustrates representative single BKCa channel recordings at −20 mV, in high and low free [Ca²⁺] (control condition) and following addition of 1 μM 20-HETE in the cis compartment (third trace). This recording shows that 1 μM 20-HETE enhanced BKCa channel activity and that this effect was completely blocked by addition of 10 nM IbTx on the extracellular side of the channel (Fig. 6A, bottom trace). Current-voltage curves obtained in control conditions and in the presence of 1 μM 20-HETE (Fig. 6B) revealed that 20-HETE had an unexpected effect on both current amplitude and unitary conductance of BKCa channels. For instance, the amplitude of the BKCa channel current at a holding potential of −20 mV was increased by 27% in the presence of 1 μM 20-HETE. Moreover, the mean conductance obtained from several BKCa channel recordings was increased from 273 ± 8 pS in control to 348 ± 12 pS in the presence of 20-HETE.

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**Fig. 3.** Effect of cyclooxygenase (COX) inhibition and COX-derived metabolite on 20-HETE-induced relaxation. **A:** paired recordings of relaxing responses induced by 3 μM 20-HETE before (control) and after 10 μM indomethacin pretreatment on bronchial rings precontracted with 1 μM MCh. **B:** bar histogram quantifying the effect of indomethacin pretreatment on 20-HETE-induced relaxation, n = 12. **C:** paired recordings of responses generated by 1 μM 20-HETE and 1 μM 20-OH-PGE₂ on bronchi precontracted with 1 μM MCh. **D:** quantitative analysis of relaxing responses to 20-HETE and two 20-OH-PGE₂ concentrations on human bronchi constricted with 1 μM MCh (n = 14). *P < 0.05 was considered as significant by ANOVA.

**Fig. 4.** Iberiotoxin (IbTx) abolishes 20-HETE relaxing responses in human bronchi. **A:** paired responses to 1 μM 20-HETE on bronchial explants precontracted with 1 μM MCh, before and after 10 nM IbTx treatment. **W,** washout. **B:** bar histogram showing the mean inhibitory effect induced by 10 nM IbTx on 20-HETE responses from bronchial tissues. Note that IbTx prevented 94% of the relaxing responses (n = 18). *P < 0.05 considered as significant by ANOVA.
Concentration-dependent effect of 20-HETE on reconstituted BKCa channels. Figure 7 illustrates a single channel recording obtained at −20 mV, at low 0.6 M free \([Ca^{2+}]\) in asymmetrical 50/250 mM KCl buffer (top trace), and following the addition of cumulative concentrations of 20-HETE (0.1, 0.3, and 1 \(\mu M\)) in the cis compartment. These unitary current recordings demonstrate the ability of the eicosanoid to activate BKCa channels in a concentration-dependent manner since 20-HETE had significant effects on channel \(P_o\) at all concentrations tested (Fig. 7B). At the maximal concentration tested, 20-HETE caused an increase in \(P_o\) from 0.05 ± 0.01 to 0.72 ± 0.09. Following data acquisition and single channel analysis of the various data files in terms of open and closed time distributions, the average mean open (\(\tau_{op1}\) and \(\tau_{op2}\), top) and closed (\(\tau_{cl1}\) and \(\tau_{cl2}\), bottom) time constants are reported in Fig. 7C. The values of the mean open times (Fig. 7C, top) show that 20-HETE significantly increased the long time constant \(\tau_{op2}\) when compared with the average values measured in low \([Ca^{2+}]\). 20-HETE also slightly increased the short \(\tau_{op1}\) open events. Furthermore, addition of 20-HETE (0.1–1 \(\mu M\)) decreased the value of long closed time constant \(\tau_{cl2}\) compared with the values determined at low \([Ca^{2+}]\) (Fig. 7B, bottom). Thus, 20-HETE appears to affect both the conducting behavior as well as the gating behavior of BKCa channels derived from human ASM cells by reducing the mean duration of shut intervals and increasing the number of long-lasting open events.

**DISCUSSION**

In this report, we investigated the ability of 20-HETE to modulate ASM tone in human bronchi. This is the first functional study assessing the effects of this eicosanoid in human bronchi. It was found that 20-HETE induces concentration-dependent and IbTx-sensitive relaxations as well as hyperpolarization of the resting membrane potential of human bronchi ASM. These effects were correlated with a direct activation of reconstituted BKCa channels from microsomal preparations.
20-HETE relaxes human bronchi. The data herein are the first to demonstrate a concentration-dependent relaxation to 10^{-8} to 10^{-5} M 20-HETE on human bronchial rings precontracted to MCh and AA. The relaxing effects of 20-HETE were also observed on resting tension and were not inhibited by PTX pretreatment, suggesting that 20-HETE does not require the involvement of a G_{i} protein to induce its relaxing effect on human ASM. The bronchodilatory effects of 20-HETE were blocked by IbTX, on the other hand, strongly suggesting that 20-HETE is an activator of BK_{Ca} channels. The relaxing effect of 20-HETE had been reported in rabbit bronchi preconstricted with histamine (2, 11, 12). This effect on rabbit ASM tissues was blocked by indomethacin or after epithelium removal. Despite the fact that the CYP-450 \omega-hydroxylase has been identified in various lung tissues (2, 30), a key issue has been to demonstrate the involvement of endogenous 20-HETE in human bronchi. Recent advances in the design of new pharmacological CYP-450 \omega-hydroxylase inhibitors, such as DDMS (5), enabled us to evaluate the putative tissue production of this eicosanoid. DDMS is indeed shown to amplify the pharmacological responses to MCh. In fact, 20-HETE is thought to play an important role in regulating bronchial tone. Although it was previously reported that the relaxing effect of 20-HETE was COX sensitive in rabbit lung (7, 12), the responses to 20-HETE were not modified by indomethacin pretreatments in human bronchi, however, as shown herein. Moreover, 20-OH-PGE_{2} had basically no relaxing effect on MCh-precontracted bronchi, suggesting that the relaxing effect induced by 20-HETE was not related to prostanoid formation. In contrast, previous studies have reported bronchoconstriction in guinea pig bronchial rings exposed to submicromolar 20-HETE concentrations (4). Differences between our observations and those reported by others are likely related to interspecies differences (28). Other eicosanoid congeners, such as epoxygenosatrienoic acid regioisomers, which are also derived from AA through the CYP-450 epoxygenase, were also shown to trigger relaxing and hyperpolarizing responses in guinea pig ASM preparations (1). With respect to the mode of actions of such eicosanoids in the control of airway tone, published data are scant, often divergent, and appear to be closely dependent on the particular tissues and species studied (28), clearly justifying the present study at the cellular and molecular level in human tissues.

20-HETE hyperpolarizes ASM cells. The intracellular microelectrode technique revealed that 20-HETE induced significant concentration-dependent hyperpolarizations of human ASM cells. Because IbTX prevented the hyperpolarizing effects and relaxation induced by 20-HETE, BK_{Ca} channel activation thus appears to be a key determinant in the regulation of human ASM tone. It has been reported that EETs also display hyperpolarizing effects in guinea pig ASM cells (1). Moreover, there is considerable evidence that eicosatetraenoic acid regioisomers (EETs), produced by the endothelium, are potent vasodilators due to their ability to activate K^{+} conductances, known to hyperpolarize VSM cells (3, 8, 15, 32). Conversely, other studies have reported that 20-HETE depolarizes VSM membrane by blocking large K^{+} conductances (10, 14) and by activating L-type Ca^{2+} currents (9).

20-HETE directly activates BK_{Ca} channels. Using the PLB reconstitution technique, we evaluated the modulation of large conducting Ca^{2+}-activated K^{+} channels by 20-HETE. This allowed us to investigate the direct action of exogenously added 20-HETE to the cis compartment (extracellular side), thus mimicking physiological release of 20-HETE by lung cells. Obtained results confirm that 20-HETE has marked effects on both the unitary conductance and gating behavior of BK_{Ca} channels by increasing the number and mean duration of the open events but decreasing the mean duration of shut intervals. These results are consistent with a direct activation of BK_{Ca} channels since they were obtained in the absence of GTP, ATP, cAMP, or other metabolites in the trans chamber (1). This mode of action of 20-HETE in ASM contrasts with the results reported in canine basilar arteries by Obara et al. (22), in which 20-HETE induced a sensitization to stretch, caused by PKC\alpha-mediated inhibition of K_{Ca} channel

Fig. 7. Concentration-dependent effects of 20-HETE on single-channel currents from reconstituted human BK_{Ca} channels. A: typical recordings of a unitary current obtained in asymmetrical 50/250 mM KCl buffer at −20 mV in low 0.6 \mu M free [Ca^{2+}] (control, top trace) and following addition of cumulative concentrations of 20-HETE (0.1–1 \mu M) in the cis compartment. B: quantiative analysis of 20-HETE effects at low 0.6 \mu M free [Ca^{2+}] on channel open probability (P_{o}). Significant P_{o} increases were obtained upon addition of 20-HETE. *P < 0.05. C: following analysis of the mean open and closed time distributions from various data files, the mean \tau_{op} (top) and \tau_{cl} (bottom) are reported in the form of bar diagrams. Subsequent addition of 20-HETE mainly resulted in a significant increase in \tau_{op} and a marked decrease in \tau_{cl}; n = 7.
activity. Thus, 20 HETE would inhibit KCa channels due to a PKC-dependent process in vascular tissues, whereas it would directly activate these channels in ASM cells. Moreover, the present results do not support and basically refute the requirement for Gα protein as well as the involvement of a PKA activation process, since experiments performed on PTX-pre-treated bronchi or in the presence of H-89 (a PKA inhibitor) showed no modification in 20-HETE relaxing responses, whereas 20-HETE was still very effective on reconstituted KCa channels from human ASM. Moreover, they suggest the existence of specific receptor binding sites on cell membrane or interactions with the protein subunits forming the octameric BKCa channels or their lipid environment. It has been reported that other AA metabolites, such as EETs, directly activate BKCa channels isolated from guinea pig ASM (1) or from human distal bronchi, as recently reported (19). Complementary experiments demonstrated that a metabolite such as 14,15-epoxy-20-HETE and AA, the precursor of 20-HETE, have no direct effect on the reconstituted KCa channel from human bronchi (data not shown). Further investigations will be required to determine whether specific binding sites are indeed present on the α- or β-subunits of the KCa channel (1). Alternatively, 20-HETE could interact with associated proteins or membrane microdomains, such as lipid rafts, or with specific phospholipid acyl chains from the lipid leaflets of the sarcoplasmic membrane of ASM cells. These hypotheses remain to be tested.

In summary, the present study provides evidence that 20-HETE is able to modulate the mechanical and electrophysiological properties of ASM in human bronchi, whereby 20-HETE induces concentration-dependent relaxations as well as ASM cell hyperpolarizations, both of which are IbTx sensitive. These events are correlated with a direct activation of BKCa channels by 20-HETE in preparations derived from human bronchi. Together, our data provide new insight into the bronchodilating action of 20-HETE in human airways, leading to speculation that this eicosanoid could represent a new pharmacological target in asthmatic and chronic obstructive pulmonary disease patients.

ACKNOWLEDGMENTS

We thank Dr. John R. Falck for the kind gift of the specific ω-hydroxylase inhibitor (DDMS), Dr. J. H. Capdevila for the gift of 14,15-epoxy-20-HETE, and Drs. Edmond Riscallah and Olivier Lesur for assistance and technical help with tissue recovery.

GRANTS

This work was supported by Canadian Institutes of Health Research Grant MOP-57677. C. Morin is the recipient of a Ph.D studentship from the Quebec Respiratory Health Network of the Fonds de la Recherche en Santé du Québec.

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