ORIGINAL RESEARCH

Active Components of Ginger Potentiate β-Agonist–Induced Relaxation of Airway Smooth Muscle by Modulating Cytoskeletal Regulatory Proteins

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Abstract

β-Agonists are the first-line therapy to alleviate asthma symptoms by acutely relaxing the airway. Purified components of ginger relax airway smooth muscle (ASM), but the mechanisms are unclear. By elucidating these mechanisms, we can explore the use of phytotherapeutics in combination with traditional asthma therapies. The objectives of this study were to: (*1*) determine if 6-gingerol, 8-gingerol, or 6-shogaol potentiate β-agonist–induced ASM relaxation; and (*2*) define the mechanism(s) of action responsible for this potentiation. Human ASM was contracted in organ baths. Tissues were relaxed dose dependently with β-agonist, isoproterenol, in the presence of vehicle, 6-gingerol, 8-gingerol, or 6-shogaol (100 μM). Primary human ASM cells were used for cellular experiments. Purified phosphodiesterase (PDE) 4D or phospholipase C β enzyme was used to assess inhibitory activity of ginger components using fluorescent assays. A G-LISA assay was used to determine the effects of ginger constituents on Ras homolog gene family member A activation. Significant potentiation of isoproterenol-induced relaxation was observed with each of the ginger constituents. 6-Shogaol showed the largest shift in isoproterenol half-maximal effective concentration. 6-Gingerol, 8-gingerol, or 6-shogaol significantly inhibited PDE4D, whereas 8-gingerol and 6-shogaol also inhibited phospholipase C β activity. 6-Shogaol alone inhibited Ras homolog gene family member A activation. In human ASM cells, these constituents decreased phosphorylation of 17-kD protein kinase C–potentiated inhibitory protein of type 1 protein phosphatase and 8-gingerol decreased myosin light chain phosphorylation. Isolated components of ginger potentiate β-agonist–induced relaxation in human ASM. This potentiation involves PDE4D inhibition and cytoskeletal regulatory proteins. Together with β-agonists, 6-gingerol, 8-gingerol, or 6-shogaol may augment existing asthma therapy, resulting in relief of symptoms through complementary intracellular pathways.

Keywords: asthma; adrenergic receptor; myosin light chain; lung; bronchodilation

Clinical Relevance

Natural herbal remedies, including ginger, have long been used to treat respiratory conditions. Many individuals with asthma use herbal therapies to self-treat their asthma symptoms; however, little is known regarding how these compounds work in the airway. In the current work, we show that 6-gingerol, 8-gingerol, and 6-shogaol potentiate β-agonist–induced relaxation of airway smooth muscle by inhibiting both phosphodiesterase 4D and phosphatidylinositol-specific phospholipase C, leading to downstream regulation of contractile proteins. These data suggest that natural compounds can work in combination with traditional asthma therapies to relieve asthma symptoms.

(Received in original form March 19, 2013; accepted in final form July 8, 2013)

This work was supported in part by National Institutes of Health grants GM065281 and GM008464 (C.W.E.).

Author Contributions: E.A.T. and C.W.E. conceived and designed the research; E.A.T., Y.Z., C.X., and R.W. performed experiments; E.A.T., Y.Z., C.X., R.W., and C.W.E. analyzed data; E.A.T. and C.W.E. interpreted results of experiments; E.A.T., R.W., and C.W.E. prepared figures; E.A.T., R.W., and C.W.E. drafted the manuscript; E.A.T., Y.Z., C.X., R.W., and C.W.E. edited and revised the manuscript; E.A.T., Y.Z., C.X., R.W., and C.W.E. approved the final manuscript.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 50, Iss 1, pp 115–124, Jan 2014

Originally Published in Press as DOI: 10.1165/rcmb.2013-0133OC on August 20, 2013
Internet address: www.atsjournals.org

The incidence and prevalence of asthma is increasing both in the United States and worldwide (10). Research efforts have been directed at better understanding disease pathogenesis and developing new therapeutics to target the main symptoms of asthma: airway hyperresponsiveness and inflammation (11). Despite these efforts, few new therapies are available to patients, and many of these patients are turning to complementary and alternative therapies to manage their symptoms (12).

Asthma is characterized by exaggerated airway narrowing and increased airway inflammation. Increased airway constriction may be a result of increased contractile signaling, impaired relaxation signaling, or a combination of both in the airway smooth muscle (ASM). To combat ASM contraction, bronchodilators are the first-line therapy during acute asthmatic exacerbations to reverse airway obstruction, primarily by relaxing ASM. Traditional asthma therapies include short- and long-acting β-agonists that induce bronchodilation by activating adenylyl cyclase, increasing 3′-,5′-cyclic adenosine monophosphate (cAMP) and activating protein kinase (PK) A (11, 13, 14); however, asthma-related deaths have been attributed to β-agonist desensitization, a direct consequence of long-acting β-agonists (13, 15–17). This highlights the need for new therapies that acutely relax contracted airways while also augmenting traditional therapies.

Among subjects with asthma, there is increasing use of herbal therapies to treat symptoms and exacerbations (3, 4, 7, 8). The use of naturally derived therapeutics for asthma began with the use of methylxanthines, including caffeine in the early 20th century (18, 19). Methylxanthines were thought to work, in part, by inhibiting phosphodiesterases (PDEs), the enzymes responsible for cyclic nucleotide degradation. To date, little is known about the mechanistic action of these and other naturally derived compounds, thus necessitating the need for detailed investigation to elucidate signaling pathways involved in airway relaxation. Current research efforts using traditional Chinese medicinal herbs showed that an extract of three plants—*Ganoderma lucidum* (Ling-Zhi), *Sophora flavescens* (Ku-Shen), *Glycyrrhiza uralensis* (Gan-Cao)—reduces lung inflammation, airway remodeling, and ASM hyperresponsiveness (20–22). These studies support our efforts to identify novel bronchodilators derived from natural sources.

We were the first group to demonstrate that purified components of the ginger root (*Zingiber officinale*) can relax human ASM and to confirm that 6-gingerol, 8-gingerol, and 6-shogoal are the active components responsible for bronchorelaxation (9). By better understanding the mechanisms by which purified components of ginger exert their effects on the airway, we can explore the use of these naturally derived phytotherapeutics in alleviating asthma symptoms alone and in combination with existing therapies. As such, we hypothesize that specific chemical components of ginger have bronchorelaxant properties and potentiate β-agonist signaling, leading to enhanced ASM relaxation.

Materials and Methods

Detailed methods are found in the online supplement.

Cell Culture

Immortalized and primary human ASM cell lines were prepared as described previously (23, 24) and grown in phenol red–free Dulbecco's modified Eagle's medium/F12 media (GIBCO, Grand Island, NY) with 10% fetal bovine serum and antibiotics.

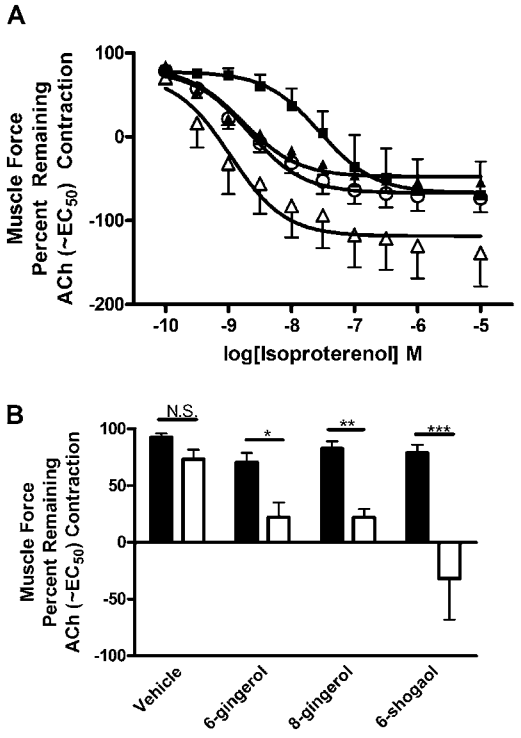

Figure 1. 6-Gingerol, 8-gingerol, and 6-shogaol potentiate isoproterenol-induced relaxation in airway smooth muscle (ASM). (*A*) In ASM strips (epithelium denuded) contracted with acetylcholine (ACh), isoproterenol elicited a concentration-dependent relaxation. Vehicle (0.2% dimethyl sulfoxide [DMSO], *solid squares*), 6-gingerol (100 μM, *solid triangles*), 8-gingerol (100 μM, *open circles*), or 6-shogaol (100 μM, *open triangles*) was added concurrently with the 300 pM concentration of isoproterenol. Each component of ginger significantly potentiated isoproterenol-induced relaxations and caused a leftward shift in the relaxation curve. Curves were fit with a four-parameter sigmoid to determine half-maximal effective concentrations (EC_{50}) summarized in Table 1 (*$P < 0.05$, n = 5–6 strips per group). (*B*) In human ASM strips contracted with ACh (∼ EC_{50}), 100 μM 6-gingerol, 8-gingerol, or 6-shogaol alone did not significantly relax tissue compared with vehicle (0.2% DMSO) within the first 7–14 minutes after addition. Moreover, the addition of 1 nM isoproterenol (ISO) to vehicle (*open bars*) did not produce significant relaxation compared with vehicle alone (*solid bars*). In tissues receiving the combination of either 6-gingerol plus ISO, 8-gingerol plus ISO, or 6-shogaol plus ISO, robust relaxation was observed that was greater than either ginger component or ISO alone (n = 5–9 strips from five patients; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). N.S., not significant.

M3 Transfection

Cells were stably transfected as previously described (25), with modifications detailed in the online supplement.

Guinea Pig Tracheal Rings

Animal protocols were approved by the Columbia University Animal Care and Use Committee. Epithelial-denuded tracheal rings were suspended in jacketed organ baths, bubbled with 95% oxygen and 5% carbon dioxide.

Human Tracheal Strips

Deidentified healthy human tracheal and bronchial ASM was obtained from discarded trimmings of donor airways from recipient lungs incident to lung transplant surgery at Columbia University (deemed not human subject research by Columbia's Institutional Review Board). Smooth muscle tissue was carefully dissected and the epithelium removed and suspended in organ baths.

Force Studies

See the online supplement for experimental details.

PDE Assay

PDE4D Assay kit (no. 60345) was obtained from BPS Bioscience (San Diego, CA) and was used according the manufacturer's instructions.

Phospholipase C β Assay

Purified phosphatidylinositol-specific phospholipase C (PI-PLC) isoform β was obtained from Life Technologies (P6466; Life Technologies, Grand Island, NY). The fluorescent indicator, 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP), was used as the enzyme substrate (D6567; Life Technologies). The enzyme (0.25 U/ml) was incubated with 6-gingerol, 8-gingerol, 6-shogaol (100 μM each), rolipram (10 μM), U-73122 (50 μM), or vehicle (2% dimethyl sulfoxide [DMSO]) for 30 minutes at room temperature. DiFMUP (100 μM) was added to the enzyme/inhibitor mix (50 μM final DiFMUP, 0.125 U/ml final PI-PLC) and the fluorescence was read every 5 minutes for 1 hour on a Flexstation3 microplate reader (358 nm excitation, 455 nm emission; Molecular Devices, Sunnyvale, CA). All comparisons were made at time = 60 minutes, and values were background corrected.

Table 1: Summary of Effects of Ginger Components of Isoproterenol Half-Maximal Effective Concentration Relaxation of an Established Acetylcholine Contraction

	Isoproterenol EC_{50} (*nM*)	
	Human ASM	**Guinea Pig ASM**
Vehicle (0.2% DMSO)	28.5 ± 2.6	8.9 ± 1.9
6-Gingerol (100 μM)	1.7 ± 0.6*	1.5 ± 0.3*
8-Gingerol (100 μM)	2.1 ± 0.5*	1.2 ± 0.2*
6-Shogaol (100 μM)	1.1 ± 0.5*	0.8 ± 0.1*
10-Gingerol (100 μM)	—	5.8 ± 2.1
U-73122 (5 μM)	—	3.2 ± 1.0

Definition of abbreviations: ASM, airway smooth muscle; DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration.
*$P < 005$ compared to vehicle.

Phosphatase Assay

Primary human ASM cell lysates were incubated with vehicle (0.1% DMSO), 6-gingerol, 8-gingerol, 6-shogaol (100 μM each), or phosphatase inhibitor cocktail (P0044, P5726; 1:100 dilution; Sigma, St. Louis, MO) for 60 minutes at room temperature in a black-walled, clear-bottom, 96-well plate. After incubation, 50 μM DiFMUP was added to each well and the fluorescence read every 5 minutes for 25 minutes on a Flexstation 3 microplate reader (358 nm excitation, 455 nm emission).

Immunoblot Analyses

Standard immunoblot techniques were used for the detection of phospho–heat shock-related protein (HSP) 20 (Ser16 no. 58522, 1:2,000 dilution; Abcam, Cambridge, MA), phospho–17-kD PKC-potentiated inhibitory protein of type 1 protein phosphatase (CPI-17; Thr 38, Abcam no. 52174, 1:2,000 dilution), myosin light chain 20 (MLC; total MLC_{20}, Abcam no. 11082, 1:10,000 dilution), phospho-MLC_{20} (Ser19; no. 3671S, 1:1,000 dilution; Cell Signaling, Danvers, MA), and β-actin (Cell Signaling no. 4970S, 1:20,000 dilution). All intensities were corrected for

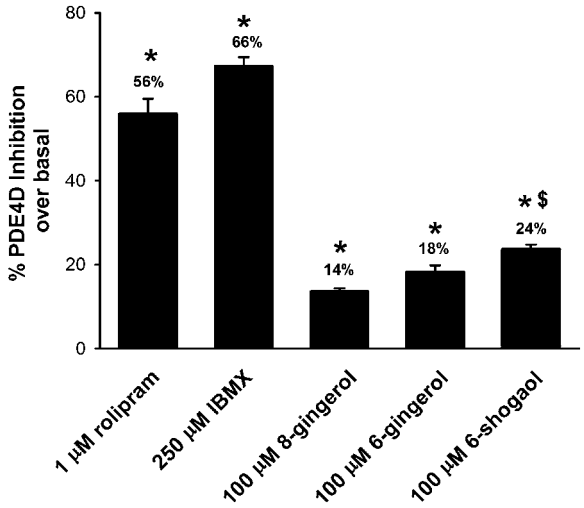

Figure 2. 6-Gingerol, 8-gingerol, and 6-shogaol inhibit phosphodiesterase (PDE) 4D. Purified PDE4D enzyme was incubated with vehicle (0.2% DMSO), rolipram (1 μM), 3-isobutyl-1-methylxanthine (IBMX; 250 μM), 8-gingerol (100 μM), 6-gingerol (100 μM), or 6-shogaol (100 μM) for 15 minutes. All compounds significantly inhibited PDE4D compared with vehicle controls (*$P < 0.01$), whereas 6-shogaol had increased inhibitory activity compared with 8-gingerol ($^{\$}P < 0.05$). Data are expressed as percent inhibition normalized to vehicle controls (n = 8–9).

protein loading (total MLC_{20} or β-actin) and quantified using densitometry (BioSpectrum Imaging System and VisionWorksLS Software UVP, Upland, CA).

Ras Homolog Gene Family Member A Activation Assay

Primary human ASM cells were grown to confluence in 60-mm dishes and serum starved for 48 hours before beginning the assay protocol (Cytoskeleton no. BK124; Cytoskeleton, Inc., Denver, CO).

Statistical Analysis

Data were analyzed using one-way ANOVA with repeated measures. Bonferroni's correction was applied for multiple comparisons. Statistical significance was established at P less than 0.05 unless otherwise noted, and all values are expressed as means (± SE).

Materials

See the online supplement for more detail on materials used.

Results

6-Gingerol, 8-Gingerol, and 6-Shogaol Potentiate β-Agonist–Induced Relaxation of Human ASM

In human ASM tissue (epithelium denuded) contracted with acetylcholine (ACh), 100 μM of 6-gingerol, 8-gingerol, or 6-shogaol showed minimal relaxation compared with vehicle controls (0.2% DMSO) within the first 7–14 minutes after addition. As such, these concentrations of the ginger constituents were used in subsequent isoproterenol potentiation studies. In separate experiments, escalating concentrations of isoproterenol (half-log increments 100 pM to 10 μM) resulted in dose-dependent relaxations with an isoproterenol half-maximal effective concentration (EC_{50}) of 28.5 nM for vehicle-treated baths. All tissues received either a single treatment of vehicle (0.2% DMSO) or 100 μM of 6-gingerol, 8-gingerol, or 6-shogaol concurrently with the 300-pM isoproterenol dose. Compared with vehicle, each active component of ginger significantly potentiated the isoproterenol-induced relaxation ($^{*}P <$ 0.05, repeated measures ANOVA). In addition, there was an observed leftward shift and decrease in the isoproterenol EC_{50} in the presence of 6-gingerol (EC_{50} = 1.7 nM), 8-gingerol (2.1 nM), or 6-shogaol (1.1 nM), with 6-shogaol being the greatest potentiator of relaxation (Figure 1A). To demonstrate that this was a synergistic effect, relaxation due to each of the ginger components alone (100 μM) measured 14 minutes after addition was compared with vehicle (0.2% DMSO), and showed no significant relaxation. In addition, 1 nM isoproterenol showed no significant relaxation compared with tissues receiving only vehicle (0.2% DMSO); however, the combination of 6-gingerol, 8-gingerol, or 6-shogaol with 1 nM isoproterenol relaxed significantly more than each of the ginger components alone (Figure 1B, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Similar results were seen in guinea pig ASM tissues contracted with ACh and subjected to identical treatment paradigms (*see* Figure E1 in the online supplement). In these studies, 100 μM 10-gingerol had no effect on isoproterenol potentiation. Similarly, the PLCβ inhibitor, U-73122 (5 μM), did not cause a significant shift in the isoproterenol EC_{50}. Results for human and guinea pig isoproterenol-induced relaxation are summarized in Table 1. The use of 10-gingerol was discontinued in all subsequent studies.

As 6-shogaol was the most robust potentiator of isoproterenol-induced relaxation, a dose–response relaxation curve with 6-shogaol alone was constructed in guinea pig ASM contracted with ACh. Maximal relaxation was observed at 300 μM, whereas vehicle exhibited a moderate increase in tone (Figure E2, $^{*}P < 0.001$ compared with vehicle; n = 5–8).

Gingerol Effects Are Not Acting by Opening K^{+} Channels

Relaxation effects of β-agonists involve, in part, large-conductance, calcium-activated potassium (BK_{ca}) channel phosphorylation,

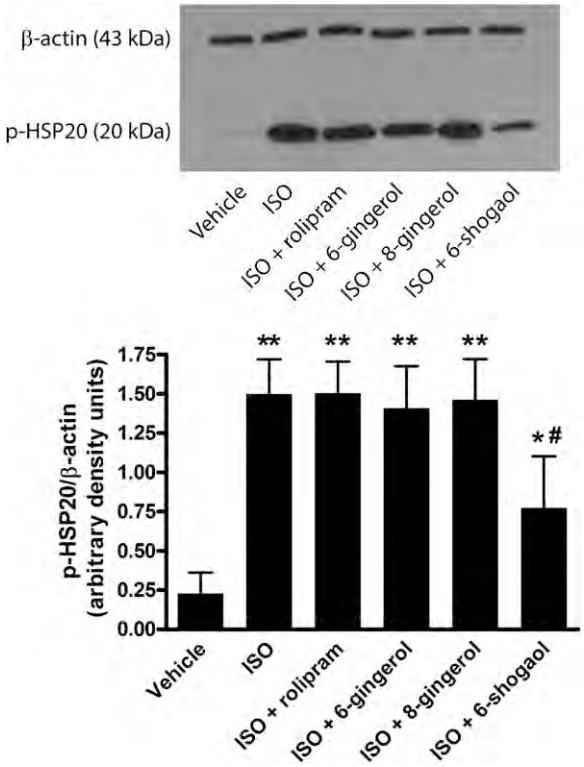

Figure 3. 6-Gingerol and 8-gingerol do not effect ISO-induced heat shock protein (HSP) 20 phosphorylation. In primary human ASM cells, 20-minute treatment with ISO (1 μM) increased phosphorylation of HSP20 (Ser 16; p-HSP20) compared with vehicle controls (0.1% DMSO). The combination of ISO with rolipram (10 μM), 6-gingerol (100 μM), or 8-gingerol (100 μM) showed no difference in phosphorylation compared with ISO alone, but was significantly increased compared with vehicle controls. The combination of ISO and 6-shogaol (100 μM) showed significant attenuation of HSP20 phosphorylation compared with ISO alone; however, this combination remained significantly increased compared with vehicle ($^{*}P < 0.05$ compared with vehicle, $^{**}P < 0.01$ compared with vehicle; $^{\#}P < 0.05$ compared with ISO alone; n = 4).

K^+ efflux, and membrane hyperpolarization. To assess if the relaxant effects of 6-gingerol, 8-gingerol, or 6-shogaol involve effects on K^+-channels, guinea pig ASM was contracted with the nonspecific K^+-channel inhibitor, tetraethylammounium (10 mM). Despite K^+ channel blockade, each active component of ginger (6-gingerol, 8-gingerol, and 6-shogaol) rapidly and significantly relaxed airway tissues (Figure E2, $^*P < 0.05$).

6-Gingerol, 8-Gingerol, and 6-Shogaol Have PDE4D-Inhibitory Action

PDEs are endogenous enzymes that degrade cAMP, the molecule that activates PKA and leads to airway relaxation. In assays using isolated, purified PDE4D enzyme (the predominant isoform in the lung and a contributor to ASM tone [26–28]), 6-gingerol, 8-gingerol, and 6-shogaol (100 μM each) exhibited enhanced PDE-inhibitory action compared with vehicle control. Rolipram (1 μM) was used as a positive control for selective PDE4 inhibition, whereas 3-isobutyl-1-methylxanthine (250 μM) was used as a nonspecific PDE inhibitor. 6-Shogaol showed the most PDE4D inhibition among the ginger constituents, and was significantly more potent than 8-gingerol (Figure 2, $^*P < 0.01$ compared with vehicle, $^\$P < 0.05$ compared with 8-gingerol).

6-Gingerol, 8-Gingerol, and 6-Shogaol Do Not Increase HSP20 Phosphorylation Akin to Other PDE4 Inhibitors or PKA Activation

In addition to phosphorylating BK_{ca} channels, PKA activation has recently been shown to phosphorylate HSP20, leading to relaxation of ASM (29, 30). In addition, PDE inhibitors alone also phosphorylate HSP20 by increasing cAMP and activating PKA independent of beta-adrenergic receptor (β-AR) activation (31). Immunoblot analyses in primary human ASM cells showed increased phosphorylation of HSP20 (Ser16) with 20 minutes of isoproterenol (1 μM) or rolipram (10 μM) compared with vehicle control (0.1% DMSO) (data not shown), confirming the results of Ba and colleagues (31). In subsequent studies, ASM cells were treated with the combination of isoproterenol (1 μM) and 6-gingerol, 8-gingerol, or 6-shogaol (all 100 μM) to approximate experimental conditions used in muscle force studies. In the presence of isoproterenol, both 6-gingerol and 8-gingerol showed no difference in HSP20 phosphorylation compared with isoproterenol alone, whereas isoproterenol treatments alone exhibited increased phosphorylation compared with basal levels. In the presence of isoproterenol, 6-shogaol attenuated HSP20 phosphorylation, but the level of phosphorylation remained significantly higher than basal levels (Figure 3, $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle, $^{\#}P < 0.05$ compared with isoproterenol alone).

6-Gingerol, 8-Gingerol, and 6-Shogaol Decrease CPI-17 Phosphorylation

Cytoskeletal regulatory proteins other than HSP20 have also been shown to regulate smooth muscle contraction and relaxation. Specifically, phosphorylation of the CPI-17 at Thr38 indirectly increases MLC_{20} phosphorylation and favors contraction by inhibiting MLC phosphatase (MLCP) (32–34). In primary human ASM cells, treatment with 10 μM ACh significantly increased CPI-17 phosphorylation compared with basal levels, whereas concurrent treatment with ACh and 6-gingerol, 8-gingerol, or 6-shogaol (100 μM; 20 min) prevented ACh-induced increases in CPI-17 phosphorylation. The Rho kinase inhibitor, Y-27632 (100 μM), was used as a positive control for attenuating ACh-induced increases in CPI-17 phosphorylation (Figures 4A and 4B, $P < 0.05$, $P < 0.01$ as indicated).

6-Shogaol but Not 6-Gingerol or 8-Gingerol Inhibit Ras Homolog Gene Family Member A Activation

In primary human ASM cells, the G protein–coupled receptor type q (G_q) agonist, bradykinin (10 μM), caused a significant increase in Ras homolog gene family member A (RhoA) activation compared with vehicle-treated controls

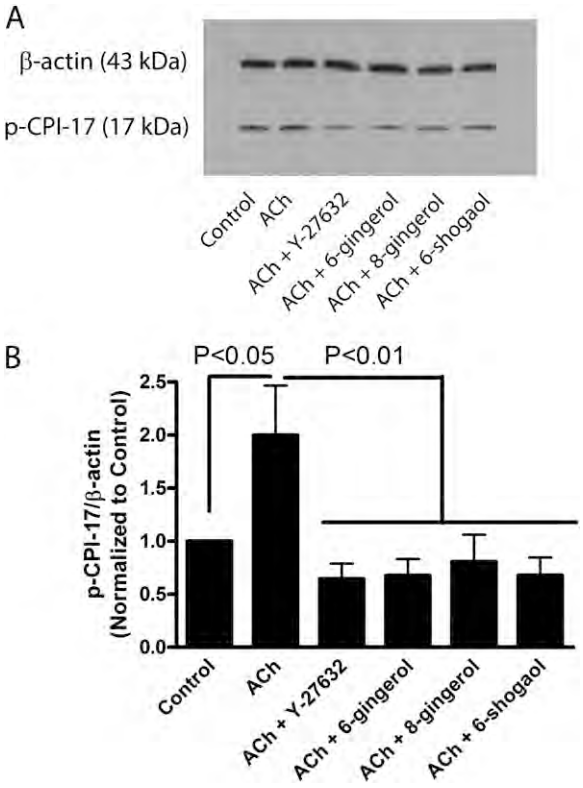

Figure 4. 6-Gingerol, 8-gingerol, and 6-shogaol attenuate 17-kD PKC-potentiated inhibitory protein of type 1 protein phosphatase (CPI-17) phosphorylation. (*A*) In primary human ASM cells, 20-minute treatment with 10 μM ACh increased CPI-17 phosphorylation (Thr 38) compared with control (0.1% DMSO, $P < 0.05$, $n = 5$). The addition of Y-27632, 6-gingerol, 8-gingerol, or 6-shogaol (100 μM each), in combination with ACh, significantly reversed the ACh-induced increase in CPI-17 phosphorylation and approximated that of vehicle controls ($P < 0.01$ compared with Ach; $n = 5$). (*B*) Summary bar graph of five experiments.

(0.2% DMSO). Pretreatment of cells for 5 minutes with 6-shogaol (100 μM) significantly abrogated this bradykinin-induced activation of RhoA, whereas 6-gingerol or 8-gingerol (100 μM each) had no effect (Figure 5A, $^{**}P < 0.01$, $^{***}P < 0.001$). Immunoblot analyses for total RhoA protein showed no differences between treatment groups (Figure 5B).

6-Gingerol, 8-Gingerol, and 6-Shogaol Do Not Increase Endogenous Phosphatase Activity

Endogenous basal phosphatase activity was measured to assess if decreased phosphorylation of HSP20 and CPI-17 was due to effects of 6-gingerol, 8-gingerol, or 6-shogaol increasing endogenous phosphatase activity in human ASM. Primary human ASM cell lysates were incubated with vehicle (0.1% DMSO), 6-gingerol, 8-gingerol, 6-shogaol (100 μM each), or a commercial phosphatase inhibitor cocktail as a positive control for 60 minutes at room temperature. After incubation, the fluorescent indicator, DiFMUP, was added to the lysates and time-lapse fluorescence was measured at 5-minute intervals. There was no difference in fluorescence between vehicle- or any of the gingerol- or shogaol-treated groups; however, the phosphatase inhibitor cocktail significantly and completely attenuated cleavage of DiFMUP and subsequently measured fluorescence (Figure E3, $^{*}P < 0.05$). These data suggest that 6-gingerol, 8-gingerol, and 6-shogaol do not appreciably inhibit endogenous phosphatases in ASM.

6-Gingerol, 8-Gingerol, and 6-Shogaol Inhibit Phosphatidylinositol-Specific PLC Activity

We have previously shown that 6-shogaol decreases inositol triphosphate (IP_3) synthesis subsequent to bradykinin stimulation in human ASM (9); however, the exact mechanism was unclear. Given the observed decrease in CPI-17 phosphorylation described here, which is activated by PKC (32), we investigated the effects of 6-gingerol, 8-gingerol, and 6-shogaol on phosphatidylinositol-specific PLCβ activity, also known as phosphatidylinositol-4, 5-bisphosphate PDE. Using purified PLCβ (0.125 U/mL) and a substrate that fluoresces on cleavage, we show that 100 μM of 6-shogaol and 8-gingerol inhibit PLCβ activity similar to the known inhibitor, U-73122 (50 μM).

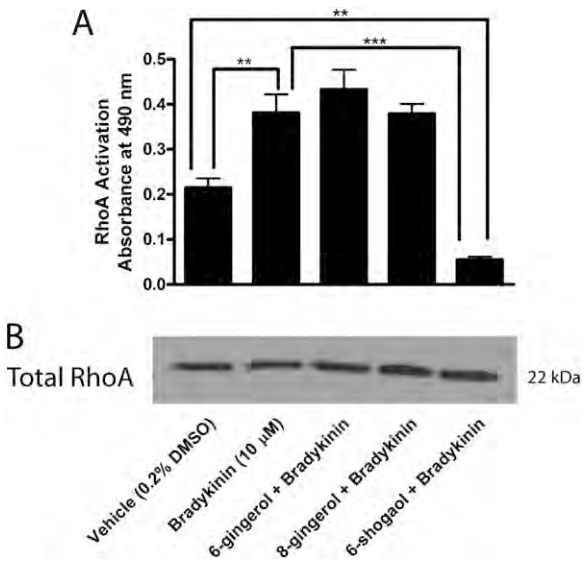

Figure 5. 6-Shogaol, but not 6-gingerol or 8-gingerol, inhibits Ras homolog gene family member A (RhoA) activation. (*A*) In primary human ASM cells, treatment with bradykinin (10 μM, 5 min) increased RhoA activity measured by G-LISA compared with vehicle controls (0.2% DMSO). A 5-minute pretreatment with 6-shogaol (100 μM) significantly abrogated bradykinin-induced increases in RhoA activity, whereas neither 6-gingerol nor 8-gingerol had an effect ($^{**}P < 0.01$, $^{***}P < 0.001$; $n = 6$). (*B*) Total RhoA protein was equivalent among all treatment groups, as determined by immunoblot.

6-Gingerol (100 μM) and the PDE4-specific inhibitor, rolipram (10 μM), were not effective at attenuating PLCβ activity (Figure 6, $^{*}P < 0.001$). These data suggest that the active components of ginger exhibit nonspecific PDE inhibition, targeting both the classical cyclic nucleotide PDEs and the phosphatidylinositol-4,5-bisphosphate PDEs, and may thus contribute to ASM relaxation.

8-Gingerol Decreases ACh-Induced Phosphorylation of MLC_{20} Likely due to Increased MLCP Activity

Phosphorylation of MLC_{20} (Ser19) by MLC kinase (MLCK) subsequent to ACh leads to

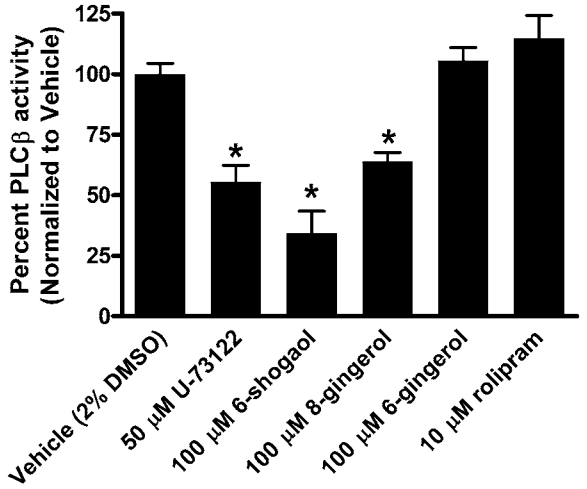

Figure 6. 8-Gingerol and 6-shogaol, but not 6-gingerol, inhibit phospholipase C (PLC) isoform β(PLCβ). Purified phosphatidylinositol-specific PLCβ was incubated with vehicle (2% DMSO), 6-gingerol (100 μM), 8-gingerol (100 μM), 6-shogaol (100 μM), rolipram (10 μM), or the commercial PLCβ inhibitor, U-73122 (50 μM), for 30 minutes. Compared with vehicle control, 6-gingerol and rolipram had no effect on PLCβ activity, whereas 8-gingerol, 6-shogaol, and U-73122 significantly attenuated PLCβ activity measured at 60 minutes ($^{*}P < 0.001$ compared with vehicle; $n = 5$–9).

smooth muscle contraction. As shown previously here, ginger constituents decrease CPI-17 activity, leading to increased MLCP activity (32, 33). Immunoblot analyses show that 8-gingerol given concurrently with ACh (100 μM) significantly attenuates ACh-induced elevations in MLC_{20} phosphorylation in M3-overexpressing human ASM cells. The Rho kinase inhibitor, Y-27632 (10 μM), was used as a positive control for reducing ACh-induced MLC_{20} phosphorylation (Figures 7A and 7B, $^*P < 0.05$).

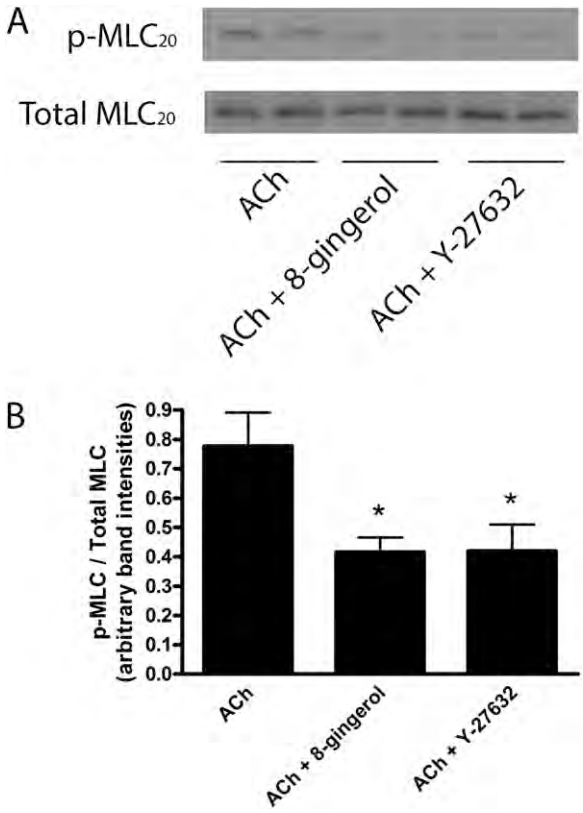

Figure 7. 8-Gingerol attenuates ACh-induced increases in myosin light chain 20 (MLC_{20}) phosphorylation. (*A*) In M3-overexpressing human ASM cells, 10-minute treatment with 100 μM ACh showed robust MLC_{20} phosphorylation (p-MLC_{20}). In ACh-treated cells, concurrent treatment with 8-gingerol (100 μM) significantly attenuated the p-MLC_{20}. The Rho kinase inhibitor, Y-27632 (10 μM), showed similar attenuation of the ACh-induced phosphorylation, and was used as a positive control. Samples were loaded in duplicate. (*B*) Summary bar graph of duplicate lanes in four separate experiments. Phosphorylated MLC_{20} was corrected for total MLC_{20} and expressed as a ratio ($^*P < 0.05$ compared with Ach-only–treated cells; $n = 4$).

Discussion

These novel data show, for the first time, that active components of ginger potentiate β-agonist–induced relaxation of human ASM. 6-Gingerol, 8-gingerol, or 6-shogaol, when given in combination with isoproterenol, exhibited a greater than 1 log shift in the isoproterenol EC_{50}, whereas 10-gingerol had no effect. Exploration into the mechanisms of action responsible for the observed potentiation showed inhibition the endogenous PDE, PDE4D, in ASM. PDE4 is a classic cyclic nucleotide PDE responsible for the degradation of cAMP, and inhibition of this enzyme leads to increased concentrations of intracellular cAMP, especially in the face of β-AR activation, leading to increased ASM relaxation. Interestingly, PLCβ is also a PDE. PLCβ cleaves phosphatidylinositol 4,5-bisphosphate at a phosphodiester bond, yielding the procontractile molecules, diacylglycerol (DAG) and IP_3. Inhibition of these two targets results in subsequent dephosphorylation of MLC_{20} and the cytoskeletal regulatory protein, CPI-17.

β-Agonist–Induced Relaxation in the Airway

The mechanisms by which cAMP regulates ASM relaxation have been extensively reviewed recently (27), and only a brief overview will be provided here. β-agonists induce bronchodilation, in part by activating adenylyl cyclase, increasing cAMP, and activating PKA. PKA phosphorylates BK_{ca} channels, leading to membrane hyperpolarization and subsequent relaxation. In addition, recent work has elucidated novel PKA targets in ASM, including the small HSP, HSP20, which contributes to relaxation (29, 31).

As more work focuses on understanding cAMP-induced bronchorelaxation, more complex and intricate signaling mechanisms are uncovered. Increased PKA activity due to increases in cAMP reduces intracellular calcium by phosphorylating IP_3 receptors on the sarcoplasmic reticulum of ASM cells (35). We previously showed that pretreatment with 8-gingerol or 6-shogaol attenuated G_q-induced increases in intracellular calcium (9). These effects may be attributed to increases in cAMP through PDE4-inhibitory actions of these compounds, leading to increased PKA activity. In 1988, Hall and Hill (36) showed that β_2-agonist stimulation can attenuate histamine-induced IP_3 accumulation in bovine ASM. Furthermore, they went on to show that the PDE inhibitors, 3-isobutyl-1-methylxanthine (1 mM) and rolipram (100 μM), also attenuated histamine-induced IP_3 accumulation; however, the mechanism was not described (37, 38). Here, we have shown, for the first time, that 6-shogaol or 8-gingerol have PDE4-inhibitory action, and also inhibit PLCβ activity directly. This inhibition of PLCβ likely explains the effect of 6-shogaol on decreased IP_3 synthesis. To our knowledge, this is the first account of a single compound that dually inhibits these two classes of PDEs, PDE4 and phosphatidylinositol-4, 5-bisphosphate PDE, in ASM. Expanding on PKA-induced smooth muscle relaxation signaling, Billington and colleagues (27) discuss the effects of PKA on inhibiting MLC phosphorylation resulting in subsequent relaxation. Here too, we show that 8-gingerol alone attenuates ACh-induced MLC_{20} phosphorylation, an effect that may also be attributed to increased cAMP

in the face of PDE4 inhibition by these compounds.

MLCK/MLCP in Contraction and Relaxation—Role for Accessory Proteins

The relative activities of MLCK and MLCP dictate the phosphorylation state of MLC_{20} and airway tone (32, 39, 40). When MLCK is activated and/or MLCP is inhibited, airway contraction is favored. When MLCK is inhibited and/or MLCP is activated, MLC_{20} is dephosphorylated and bronchodilation is observed. It is becoming increasingly evident that accessory proteins that modulate MLCK and MLCP phosphorylation states help to determine airway tone, often times independent of changes in intracellular calcium. In the current studies, we have examined MLC_{20} phosphorylation, phosphorylation of both HSP20 and CPI-17, as well as RhoA activation in the presence of 6-gingerol, 8-gingerol, or 6-shogaol (summarized in Figure 8).

A previously reported method of airway relaxation involving accessory proteins includes phosphorylation of HSP20 by PKA (reviewed in Ref. 30). Our current data suggest that HSP20 phosphorylation by 6-gingerol, 8-gingerol, or 6-shogaol alone is not a mechanism to explain the observed potentiation of β-agonist–induced relaxation. In addition, it suggests that HSP20 phosphorylation in itself is sufficient, but not necessary, to induce ASM relaxation.

In separate studies, Boterman and colleagues (41) found potentiation of β-AR function in tracheal smooth muscle by inhibiting PKC, whereas Nakahara and colleagues (42) found similar potentiation with Rho kinase inhibition. CPI-17 is a downstream target of both PKC and Rho kinase in ASM (43). CPI-17 inhibits MLCP and leads to MLC_{20} phosphorylation and subsequent contraction. By decreasing CPI-17 phosphorylation, the inhibitory action of this protein on MLCP is removed and relaxation is favored. The potentiation observed by Boterman and colleagues and Nakahara and colleagues could be attributed to decreased CPI-17 phosphorylation downstream of PKC or Rho kinase inhibition. Recently, Mukherjee and colleagues (44) found that PKC activation in the airway leads to CPI-17 phosphorylation and increases in MLC_{20} phosphorylation. Here, we have shown that 6-gingerol, 8-gingerol, and 6-shogaol prevent ACh-induced phosphorylation of CPI-17. PLCβ is an upstream enzyme leading to PKC activation that is inhibited by these compounds. In addition, 6-shogaol prevents G_q-induced activation of RhoA, which would further explain decreased CPI-17 phosphorylation. A recent review by Wright and colleagues (43) noted a correlation between CPI-17 expression and activity in both rat models of allergic asthma as well as in airway tissues from patients with asthma. This suggests a functional role for CPI-17 in the disease state, but also presents a unique target to combat airway hyperresponsiveness.

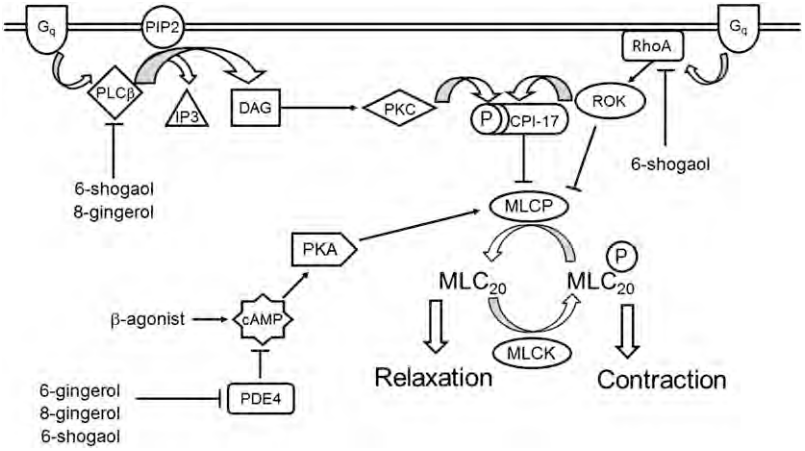

Figure 8. Isolated components of ginger, 6-gingerol, 8-gingerol, and 6-shogaol, have multiple intracellular targets that potentiate β-agoinist–induced relaxation in ASM. 6-Gingerol, 8-gingerol, and 6-shogaol inhibit PDE4D, thereby increasing the amount of intracellular 3′-,5′-cyclic adenosine monophosphate (cAMP) and increasing protein kinase (PK) A activation. In addition, 8-gingerol and 6-shogaol inhibit PLCβ, thereby decreasing inositol triphosphate and DAG synthesis, the latter of which decreases PKC activation and subsequent CPI-17 phosphorylation. Decreased CPI-17 phosphorylation removes MLC phosphatase (MLCP) inhibition, leading to MLC_{20} dephosphorylation and net relaxation. 6-Shogaol prevents RhoA activation, further decreasing CPI-17 phosphorylation. DAG, diacylglycerol; G_q, G protein–coupled receptor type q; PIP2, phosphatidylinositol4,5-bisphosphate; PLCβ, PLC isoform β (phosphatidlyinositol-4,5-bisphosphate PDE); MLCK, MLC kinase.

Ubiquitous PDE Inhibitors

The use of natural compounds to increase cAMP is not a new concept. Methylxanthines were used to relieve asthma symptoms, and theophylline, a nonspecific PDE inhibitor, was an early asthma therapeutic (18). We have shown, for the first time, that the active components of ginger, 6-gingerol, 8-gingerol, and 6-shogaol, have PDE4D-inhibitory action, and that 8-gingerol and 6-shogaol also inhibit PLCβ. Typically, PDE inhibitors are thought to inhibit the cyclic nucleotide PDEs that degrade cAMP and/or cGMP. However, it is important to note that PLCβ is also an endogenous PDE (phosphatidylinositol-4,5-bisphosphate PDE), and nonspecific PDEs may also inhibit PLCβ, as was found in the present study for 8-gingerol and 6-shogaol. Interestingly, the PDE4-specific inhibitor, rolipram, as well as 6-gingerol had no effect on PLCβ activity. Working via increasing cAMP via PDE4D inhibition and attenuating IP_3 and DAG production through PLCβ inhibition, these compounds target two signaling pathways that favor relaxation in ASM.

What This Means for β_2-AR Desensitization and Future Therapeutics

The reliance on short- and long-acting β-agonists to manage asthma symptoms and exacerbations can lead to receptor desensitization and down-regulation. This increases the risk for asthma-related death and necessitates the development of novel therapeutics that can: (*1*) decrease the reliance on β-agonists by potentiating their bronchodilating effects at lower effective concentrations; and (*2*) work to relax ASM

by complementary yet alternative signaling pathways. We have shown that active components of ginger can achieve both of these objectives by inhibiting cAMP degradation in ASM, preventing IP_3 and DAG generation, and thereby modulating accessory proteins that regulate contractile machinery within the cell. This has the potential to decrease reliance on β-agonists and help preserve β_2-AR expression and activity in the airway. Dixon and Santana (40) recently asked the question, "does inhibition of PKC in ASM increase airflow during asthma and COPD?" Our current data, together with our previous *in vivo* studies (9), argue that this is a potential signaling mechanism to explain the bronchorelaxant properties of 6-gingerol, 8-gingerol, and 6-shogaol, and may prove a yet-unrealized target for future asthma therapies. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. William Gerthoffer for the generous gift of immortalized human airway smooth muscle cells.

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