Regulatory Haplotypes in *ARG1* Are Associated with Altered Bronchodilator Response

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Rationale: β_2 -agonists, the most common treatment for asthma, have a wide interindividual variability in response, which is partially attributed to genetic factors. We previously identified single nucleotide polymorphisms in the arginase 1 (*ARG1*) gene, which are associated with β_2 -agonist bronchodilator response (BDR).

Objectives: To identify cis-acting haplotypes in the *ARG1* locus that are associated with BDR in patients with asthma and regulate gene expression *in vitro*.

Methods: We resequenced *ARG1* in 96 individuals and identified three common, 5' haplotypes (denoted 1, 2, and 3). A haplotype-based association analysis of BDR was performed in three independent, adult asthma drug trial populations. Next, each haplotype was cloned into vectors containing a luciferase reporter gene and transfected into human airway epithelial cells (BEAS-2B) to ascertain its effect on gene expression.

Measurements and Main Results: BDR varied by haplotype in each of the three populations with asthma. Individuals with haplotype 1 were more likely to have higher BDR, compared to those with haplotypes 2 and 3, which is supported by odds ratios of 1.25 (95% confidence interval, 1.03–1.71) and 2.18 (95% confidence interval, 1.34–2.52), respectively. Luciferase expression was 50% greater in cells transfected with haplotype 1 compared to haplotypes 2 and 3.

Conclusions: The identified *ARG1* haplotypes seem to alter BDR and differentially regulate gene expression with a concordance of decreased BDR and reporter activity from haplotypes 2 and 3. These findings may facilitate pharmacogenetic tests to predict individuals who may benefit from other therapeutic agents in addition to β_2 -agonists for optimal asthma management.

Clinical trial registered with www.clinicaltrials.gov (NCT00156819, NCT00046644, and NCT00073840).

Keywords: pharmacogenetics; asthma; β_2 -agonist

Asthma is a complex disease characterized by chronic inflammation and constriction of bronchial smooth muscles that is variably reversible by β_2 -agonists. Asthma is estimated to affect 300 million individuals worldwide and its prevalence is predicted to increase to 400 million by 2025 (1). Despite the availability of several classes of therapeutic agents for asthma treatment, up to 50% of patients do not benefit from one or

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Asthma pharmacogenetic studies of variable β_2 -agonist response to date have identified several single nucleotide polymorphisms. However, these collectively account for only a small fraction of the phenotypic variability and the direction of some of these associations differs across populations. Additional variants likely contribute to the interindividual variability in drug response, and replication of these genetic associations and experimental data demonstrating their functional significance will improve the understanding of variable bronchodilator responses and facilitate individualized asthma therapy.

What This Study Adds to the Field

This study identifies three common haplotypes in the *ARG1* locus that are associated with β_2 -agonist response in three independent populations with asthma and are shown to differentially regulate gene expression *in vitro*.

more of these therapies (2, 3). The most commonly used class of medications for asthma treatment are short-acting β_2 -agonists, which are fast-acting reliever drugs that target acute bronchoconstriction (4). These agents activate β_2 -adrenergic receptors on airway smooth muscle cells, which stimulate cyclic adenosine monophosphate production, evoking relaxation and bronchodilation.

In an ongoing effort to identify genetic variants that alter β_2 -agonist responsiveness, we previously identified noncoding, single nucleotide polymorphisms (SNPs) located 5' of the gene encoding arginase 1 (*ARG1*) that are associated with bronchodilator response (BDR) (5). Some of these and additional polymorphisms in *ARG1* have been correlated with asthma risk (6) and atopy (7). Furthermore, earlier studies have reported higher arginase expression in the airways of people with asthma (8, 9), and a correlation between increased arginase expression and reduced lung function (10). Finally, inhibition of *ARG1* expression in the lung greatly diminished airway response to methacholine and proinflammatory cytokines (11). Based on these findings, we hypothesized that genetic variants in *ARG1*, leading to a decrease in gene expression or activity, predispose individuals to low BDR.

To test this hypothesis, we began by resequencing the *ARG1* locus to identify additional genetic variants and ascertain the most prevalent haplotypes (combination of alleles at multiple

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nucleotide positions). These haplotypes were then tested for association with BDR among patients with asthma, and were subsequently evaluated in transfection experiments using airway epithelial cells to ascertain expression phenotypes by haplotype. Some of the results of this study have been previously reported in the form of an abstract (12).

METHODS

Study Populations

DNA for resequencing of *ARG1* was obtained from 96 individuals (40 non-Hispanic whites, 32 African Americans, and 24 Hispanics), ascertained from the NHLBI Severe Asthma Research Program population (13) and from the Collaborative Study of the Genetics of Asthma (14). DNA for the genetic association study were extracted from three adult populations with asthma composed primarily of non-Hispanic white adults with mild to severe asthma: (1) the Sepracor study ($n = 435$) was a medication trial conducted by Sepracor, Inc., (15, 16); (2) the Leukotriene Modifier or Corticosteroid Salmeterol (LOCCS) study ($n = 159$); and (3) the Effectiveness of Low Dose Theophylline as an Add-on Treatment in Asthma (LODO) trial ($n = 155$). Both LOCCS and LODO were drug trials by the American Lung Association Asthma Clinical Research Centers (17). Details of each population are included in the online supplement. Each study was approved by the Institutional Review Board of the relevant institutions and all participants provided informed consent for ancillary genetic testing.

Resequencing and Genotyping

We resequenced the 5' region (~2.5 kb), 3' untranslated region, and exons of *ARG1* in the resequencing cohort (see online supplement). In the adult populations with asthma, four 5' SNPs that tag the three major haplotypes were genotyped using a SEQUENOM MassARRAY MALDI-TOF mass spectrometer (Sequenom, San Diego, CA). Each SNP had a completion rate of greater than 95% and a Hardy-Weinberg equilibrium P value of greater than 0.01.

Statistical Methodology

BDR was quantified as the percent change in FEV₁ after administration of two inhalations (totaling 180 μ g) of albuterol via a metered dose inhaler ($BDR = 100 \times [\text{postFEV}_1 - \text{preFEV}_1 / \text{preFEV}_1]$). Because of ascertainment differences and variability in BDR distribution across the three populations (Table 1), BDR was dichotomized by the median value in each asthma trial ($BDR \geq \text{median} = 2$, otherwise = 1). Single SNP association analyses used PLINK (v1.07; <http://pngu.mgh.harvard.edu/purcell/plink/>) (18) and haplotype analyses was conducted using the Haplo.stats package in R (19). The odds ratio (OR) of each haplotype by population and summary OR were determined using the random effects (DerSimonian-Laird) meta-analysis function of the Rmeta package in R (20). Additional information is available in the online supplement.

Luciferase Reporter Assays

The 5' genomic region of *ARG1* (3548 bp) was amplified (see online supplement) using DNA from three subjects, with one or two copies of each common haplotype. These amplicons were cloned into the pGL4.10[LUC2] vector (Promega) and validated by sequencing. The region 5' of ~2.5 kb was identical among all constructs. These vectors were then transiently transfected into the immortalized human airway epithelial cell line BEAS-2B and grown as previously described (21). These cells were cotransfected with pGL4.75[hRLUC/CMV] (Promega) consisting of the *Renilla* luciferase and cultured with and without (10 μ M) isoproterenol. Additional control studies were also performed with the empty pGL4.10[LUC2] vector, which provides quantitation of the background reporter luciferase activity in the absence of a promoter. Results are presented as the ratio of *LUC2* to *Renilla* luciferase and expression differences by haplotype were calculated using a two-way Student t test.

TABLE 1. BASELINE CHARACTERISTICS OF THREE ADULT ASTHMA TRIAL POPULATIONS

	LOCCS ($n = 159$)	LODO ($n = 155$)	Sepracor ($n = 435$)
Age, mean yr (SD)	35.3 (15.1)	42.9 (14.7)	32.4 (13.6)
Range	7–71	15–76	12–80
Male, n (%)	54 (34)	39 (25.2)	214 (49.2)
Prebronchodilator FEV ₁ % predicted,* mean (SD)	84.3 (12.4)	78.2 (16.5)	61.5 (6.8)
BDR			
Mean, % (SD)	6.3 (6.1)	9.7 (11.1)	40.3 (20.6)
Median	5.9	7.5	35.6
Skewness	0.04	1.3	1.5
Kurtosis	0.4	5.9	3.1

Definition of Abbreviations: BDR = bronchodilator response; LOCCS = Leukotriene Modifier or Corticosteroid Salmeterol study; LODO = Effectiveness of Low Dose Theophylline as an Add-on Treatment in Asthma study.

* Prebronchodilator FEV₁% predicted = (prebronchodilator FEV₁/predicted FEV₁) $\times$ 100%.

RESULTS

Resequencing of *ARG1* revealed a total of 22 polymorphisms with variable minor allele frequencies among the non-Hispanic whites, African Americans, and United States Hispanic subjects (see Table E1). Nine of these variants have not been previously described in dbSNP (denoted as novel) and only one synonymous polymorphism was found in an exon, encoding amino acid asparagine at position 90. Table E2 lists the 14 polymorphisms identified in the 1.7 kb region located immediately 5' of the initiator codon methionine. These 5' SNPs were found to exist in 10 haplotypes, three of which had frequencies greater than 5% and combine to represent between 80 and 91% of the population (Table E2). Four SNPs that tag these three haplotypes (denoted 1, 2, and 3 in order of frequency) were genotyped in the three populations with asthma.

Characteristics of the asthma trials are detailed in Table 1. These populations vary in the distribution of BDR, which is reflective of the variable ascertainment criteria of the independent studies. For example, the mean BDR of LOCCS is lower compared with the other trials and the LOCCS BDR distribution is more normal (i.e., skewness of 0.038 and kurtosis of 0.444). Given the phenotypic variability across our asthma trial populations, we did not apply conventional clinical thresholds of BDR for classifying patients as "responders" ($\geq 12\%$ BDR) (15, 16). Instead, the median BDR value of each trial was used to differentiate between high and low responders within each drug trial. Other distinctions among the trials include the sex composition, with Sepracor having the largest proportion of males compared to LOCCS and LODO.

Despite minor differences in haplotype frequencies among the clinical trial populations (Table 2) and the resequencing cohort (Table E2), the order by frequency of the three major haplotypes was identical in each population. Deviations in haplotype frequency may be explained by the fact that the resequencing cohort consisted of cases of asthma and unaffected controls, whereas the asthma drug trials included cases with mild to severe asthma. In addition, there were differences in sample size with the asthma drug trials consisting of a larger number of participants than the resequencing cohort. Finally, the less common haplotypes identified in the resequencing cohort were tagged by rare and several novel SNPs that were not genotyped in the asthma trial populations. The frequency of each haplotype, however, was consistent across the three adult asthma trials.

Association analyses of the individual SNPs with BDR indicated significant associations between genetic variants in

TABLE 2. HAPLOTYPE FREQUENCIES AND SINGLE SNP ASSOCIATION ANALYSES IN THREE ASTHMA TRIALS

Haplotype	SNPs				Haplotype Frequency		
	rs2781659	rs2781663	rs2781665	rs60389358	LOCCs (n = 159)	LODO (n = 155)	Sepracor (n = 435)
1	A	T	A	C	0.66	0.67	0.65
2	G	A	T	C	0.20	0.20	0.21
3	G	A	T	T	0.14	0.12	0.12
Study	Single SNP Association P value*						
LOCCs	0.01	0.01	0.01	0.04			
LODO	0.20	0.23	0.07	0.05			
Sepracor	0.05	0.03	0.02	0.01			

* P values derived from logistic regression analysis of BDR using the additive model and after adjustments for age, sex, and height.

ARG1 and BDR (Table 2) in LOCCS and Sepracor (P values ≤ 0.05) but a modest association in LODO (P values ranging from 0.05–0.23). Whereas three of the SNPs have been previously associated with BDR among people with asthma (5) and are in strong linkage disequilibrium (LD) with each other ($r^2 = 0.93$ and $r^2 = 0.98$), the fourth SNP, rs60389358, has not been associated with BDR previously and is not in LD with the other SNPs (max $r^2 = 0.28$).

Association analyses of the haplotypes with BDR, dichotomized by the median value in each trial population, yielded significant global P values (i.e., across haplotypes) in LODO and Sepracor ($P = 0.03$) and nominal association in LOCCS ($P = 0.06$) (Table 3). Haplotype-specific analyses revealed that haplotype 1 was associated with high BDR as indicated by the positive scores, whereas haplotype 3 was associated with low BDR (denoted by negative score values). These haplotype-specific associations were significant in the LOCCS and Sepracor trials (P values < 0.04) but not significant in LODO. The score values and specific P values derived for haplotypes 1 and 3 in LODO were consistent with the results of the other populations; however, the P values were short of significance ($P = 0.15$ for haplotype 1 and $P = 0.07$ for haplotype 3). Finally, haplotype 2 was not significantly associated with BDR in any of the populations but there was a trend toward a correlation with low BDR (indicated by negative score values).

Haplotype analyses of BDR as a continuous trait were consistent with the binary results in that both demonstrated a positive correlation (indicated by positive haplotype-specific score values) with haplotype 1 and a negative correlation with haplotype 3 across all three asthma trials (Table E6). Furthermore, the magnitude of haplotype effects (Global stat and haplotype-specific scores) was comparable between the two phenotypic outcomes. In addition, stronger haplotype-specific associations were derived for LODO using the continuous BDR variable compared with the binary trait. However, associations in Sepracor were not significant across all haplotypes (global statistics) and for haplotype 1.

In all three populations, individuals with haplotype 1 were more likely to respond to β_2 -agonists than those with haplotype 2 (summary OR = 1.25 [95% confidence interval, 0.96–1.63]) and even more likely to respond than patients with haplotype 3 (summary OR = 2.18 [95% confidence interval, 1.55–3.05]) (Figure 1, Table E3). These effect sizes were not heterogeneous among populations (DerSimonian-Laird random effects P values of 0.70 and 0.89 for haplotype 1 vs. 2 and haplotype 1 vs. 3, respectively). In general, the haplotype effect was greater than that of each individual SNP (Figure E1, Table E5), although the difference was small for rs60689358.

To ascertain whether the *ARG1* haplotypes differentially affect gene expression, the 5' region was amplified from genomic DNA of subjects bearing at least one copy of each of the three

haplotypes and amplicons were cloned into an expression vector upstream of a luciferase reporter gene. The human airway epithelial cell line BEAS-2B was chosen as the host cell. As shown in Figure 2A, these cells endogenously express *ARG1* as indicated by Western blots. Furthermore, luciferase activity from BEAS-2B cells that were transfected with haplotype 1 fused to the luciferase reporter gene was approximately 10-fold greater than those transfected with the promoter-less (empty) luciferase construct (Figure 2B). BEAS2B were transfected with equal amounts of the three plasmids bearing one of the three haplotypes with the reporter, and luciferase expression was ascertained 24 hours post-transfection. As shown in Figure 3A, haplotype 1-transfected cells had the greatest luciferase expression compared with haplotype 2 and haplotype 3 ($P < 0.001$). This difference amounted to approximately 50% greater expression for haplotype 1. Luciferase expression also differed between haplotypes 2 and 3 ($P = 0.04$), but the magnitude of this difference was small. Additional studies examined the effects of treating these transfected cells with the β_2 -agonist isoproterenol on gene regulation. Although there was a trend toward haplotype 1 cells exhibiting greater isoproterenol-promoted down-regulation, this did not reach statistical significance and the magnitude of the difference by haplotype was small (Figure 3B).

DISCUSSION

This article describes polymorphisms located 5' of *ARG1* that regulate gene expression and are associated with BDR in three populations with asthma. In the 5' region spanning approximately 1.7 kb, we identified a total of 14 polymorphisms via resequencing of *ARG1*. The most common haplotype (denoted

TABLE 3. HAPLOTYPE ASSOCIATIONS WITH BDR IN THE THREE ASTHMA TRIAL POPULATIONS USING THE ADDITIVE MODEL AND ADJUSTED FOR AGE, SEX, AND HEIGHT

Cohort	Haplotypes							
	Global Stat*	Global P*	1		2		3	
			Score†	P Value†	Score	P Value	Score	P Value
LOCCS	7.57	0.06	2.30	0.02	−0.79	0.43	−2.10	0.04
LODO	9.06	0.03	1.43	0.15	−0.86	0.39	−1.84	0.07
Sepracor	9.05	0.03	2.10	0.04	−0.06	0.95	−2.96	0.003

Definition of Abbreviations: BDR = bronchodilator response; LOCCS = Leukotriene Modifier or Corticosteroid Salmeterol study; LODO = Effectiveness of Low Dose Theophylline as an Add-on Treatment in Asthma study.

* Global statistics indicates the overall association between the haplotypes and BDR.

† Haplotype-specific statistics test the association of each individual haplotype with BDR whereby positive scores indicate a positive correlation with the phenotypic outcome.

Figure 1. Odds ratios (OR) indicate greater likelihood of a high β_2 -agonist BDR for haplotype 1. (A) Summary OR = 1.25 for haplotype 1 versus haplotype 2. (B) Summary OR = 2.18 for haplotype 1 versus haplotype 3. Boxes represent the point estimate for each study, the width of which is proportional to the standard error. The summary OR is represented as a diamond, the width of which is proportional to the standard error. Horizontal lines represent 95% confidence intervals.

1) at this locus is associated with higher BDR in all trials, whereas the two less frequent haplotypes correlate with low BDR. Furthermore, these haplotypes were shown to differentially regulate luciferase expression in our reporter gene assays with the major haplotype increasing reporter gene expression. Thus, our results demonstrate that a combination of alleles at multiple 5' SNPs regulate gene expression and modulate an individual's response to β_2 -agonists. Specifically, haplotype 1 was shown to increase gene expression by 50% compared with the other two haplotypes and was associated with a 25–118% greater likelihood of a high response to β_2 -agonists.

Although increased arginase activity was first detected among patients with asthma nearly two decades ago (8), its role in asthma susceptibility remains poorly understood and the mechanism whereby genetic variants in the arginase pathway might lead to interindividual differences in BDR is unknown. However, earlier studies have demonstrated that increased expression of arginase results in increased production of L-ornithine and decreased nitric oxide (NO) levels as a result of competitive binding of arginase and nitric oxide synthase (NOS) to the common substrate L-arginine (22, 23). L-ornithine is known to be a key element in tissue repair and airway remodeling (24), whereas NO is known to regulate inflammation and smooth muscle relaxation (9). Decreased NO levels have been shown to induce production of peroxynitrites, which are proinflammatory, cytotoxic, and stimulate airway smooth muscles to contract (25). These results were consistent with other studies showing that reduced NO levels promote increased airway reactivity to methacholine (26, 27) and cytokines (11), and increased response to repeated allergen challenge (28). Not surprisingly, elevated arginase activity has also been correlated with reduced lung function (10). Nevertheless, the link between β_2 -agonist bronchodilation and arginase 1 activity remains undefined.

In our transfected cells, we found little or no haplotype-dependent effects of β_2 -agonist on luciferase expression, suggesting that there are no receptor-activated changes in transcription factors that regulate *ARG1* expression under these experimental conditions. Other conditions or cell types might reveal differential effects of β_2 -agonist on promoter activity. Indeed, the complement of transcription factors, and their regulation by β_2 -agonist, differs substantially between airway epithelial cells and smooth muscle cells (21). However, cells containing haplotype 1 expressed significantly greater reporter gene activity compared with cells containing the other two

haplotypes. Furthermore, haplotype 1 showed the strongest association with high BDR in this manuscript.

The potential interaction between the arginase 1 and β_2 -adrenergic receptors pathways may be primarily at the physiologic level, with NO and cyclic adenosine monophosphate both having independent effects on smooth muscle relaxation. If so, one might expect subjects with haplotype 1 to have less NO, increased smooth muscle tone, and thus a greater change in β_2 -agonist-mediated flow as a consequence of the increased tone. However, the FEV_{1%} predicted for patients with haplotype 1 was not decreased, as would be expected in this scenario (Table E4). Finally, we recognize that unexpected regulation of seemingly “unrelated” pathways is often found using unbiased approaches, such as expression microarrays. We have observed such crosstalk when specific signal transduction proteins are knocked-down or overexpressed in airway smooth muscle cells (29–31) so we cannot exclude an interaction between the two pathways at this time.

Figure 2. BEAS-2B cells express *ARG1* and the *ARG1* promoter displays enhanced luciferase reporter activity. (A) Western blot of BEAS-2B cells extract reveals a band at 34.7 kD consistent with endogenous expression. Cells transfected with human *ARG1* show enhancement of this signal at the expected molecular weight. (B) BEAS-2B cells were transfected with LUC2 empty vector or *ARG1*(Hap1)-LUC2, and luciferase activity determined. $P < 0.001$ versus LUC2 empty vector. Results were from four experiments

Figure 3. The three most common haplotypes in the promoter region of the ARG1 gene differentially regulate the expression of a luciferase reporter gene. Haplotypes were cloned 5' of a luciferase reporter gene and transfected into the human airway epithelial cell line BEAS-2B. (A) Expression was different based on haplotype. Shown are results from 10 experiments. * $P < 0.001$ versus haplotype 1; # $P = 0.039$ versus haplotype 2. (B) β_2 -agonist (isoproterenol) had equivalent effects in minor down-regulation of the promoter haplotypes. Results were from six experiments.

A limitation of our study was the small sample sizes of our trial populations, especially for LOCCS ($n = 159$) and LODO ($n = 155$), which precluded integration of low-frequency haplotypes identified through the ARG1 sequencing. In future studies, the integration of large populations that homogeneously span a wide range of BDR may help to improve the understanding of the effects of ARG1 haplotypes on BDR.

The haplotypes we have identified in ARG1 have the potential to facilitate the development of genetic tests for BDR prediction in those with asthma. In addition, this study suggests that arginase 1 may be an important therapeutic target for asthma treatment. It has been suggested that L-arginine supplementation would benefit patients with asthma by increasing substrate availability for NOS to elevate the bronchodilator NO (9, 32). Alternatively, previous studies have also suggested that arginase inhibition would be beneficial to patients with asthma. For example, a loss of function of arginase 1 by RNA interference resulted in decreased airway response to methacholine and decreased response to IL-13 (11), whereas the use of an arginase inhibitor led to a decrease in airway

hyperresponsiveness after an allergic reaction (32). In contrast to these findings, Ckless and coworkers (33) reported an increase in airway hyperresponsiveness among mice administered with a different arginase inhibitor. Moreover, Niese and colleagues (34) demonstrated that arginase is not required for regulation of inflammation and hyperresponsiveness of the lungs in chimeric mice deficient of arginase 1. These conflicting results may be explained by the different methods used for arginase inhibition (i.e., RNAi, inhibitors, genetic ablation of bone marrow arginase); nonspecific inhibition of arginase via these approaches; and the different animal models used (i.e., human vs. mice vs. guinea pigs). Collectively, these earlier reports and the current study suggest that further investigation to gain a better understanding of arginase regulation is necessary to determine how modifying this pathway would improve asthma therapy.

In conclusion, we have identified haplotypes in the ARG1 locus that are associated with BDR to β_2 -agonists in three independent asthma cohorts. *In vitro* studies with transfected cells revealed differential reporter gene expression by haplotype. Depending on ethnicity, the lower-responding haplotypes may represent approximately 25–50% of the population, which is sufficient to consider ARG1 promoter haplotyping as part of a collection of pharmacogenetic tests to individualize asthma therapy. However, the treatment options for those with the unfavorable haplotypes are not known and require specific clinical trials.

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