Leukotriene B₄ production in healthy subjects
carrying variants of the arachidonate
5-lipoxygenase-activating protein gene
associated with a risk of myocardial infarction

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ABSTRACT

Leukotrienes are implicated in the pathogenesis of coronary artery disease. Recently two haplotypes (HapA and HapB) in the gene encoding ALOX5AP (arachidonate 5-lipoxygenase-activating protein), the main regulator of 5-lipoxygenase, have been associated with a doubling of the risk of myocardial infarction. Studies have also shown that treatment with a leukotriene inhibitor reduces biomarkers of coronary risk in patients carrying HapA, raising the possibility of developing genotype-specific therapy. In the present study, we examined whether carriage of HapA or HapB is associated with increased LTB₄ (leukotriene B₄) production in healthy subjects. Age- and gender-matched healthy HapA carriers (n = 21), HapB carriers (n = 20) and non-A/non-B carriers (n = 18), with no reported history of cardiovascular disease, were recruited following DNA screening of 1268 subjects from a population-based study. Blood neutrophils were isolated, and LTB₄ production was measured in response to stimulation with 1 µmol/l of the calcium ionophore A23187. There was no difference in the mean level for LTB₄ production in the three groups (non-A/non-B, 24.9 ± 8.3 ng/10⁶ cells; HapA, 22.2 ± 11.9 ng/10⁶ cells; HapB, 19.8 ± 4.8 ng/10⁶; P = 0.14). The findings indicate that if either the HapA or the HapB haplotype of ALOX5AP indeed increases cardiovascular risk, then the mechanism is not simply due to a systematically observable effect of the haplotype on LTB₄ production in response to stimulation. The results suggest that knowledge of a patient’s haplotype may not provide useful information on the probable clinical response to ALOX5AP inhibitors.

INTRODUCTION

MI (myocardial infarction) is a complex disease resulting from the interaction of environmental and genetic factors. In the majority of subjects, coronary atherosclerosis, an inflammatory process, provides the substrate for the condition and MI develops when the atherosclerosis is complicated by plaque fissuring and thrombosis [1]. LTs (leukotrienes) are inflammatory mediators derived from arachidonate by the 5-lipoxygenase pathway [2], which

Key words: arachidonate 5-lipoxygenase-activating protein (ALOX5AP), cardiovascular risk, leukotriene, myocardial infarction, single nucleotide polymorphism.

Abbreviations: ALOX5AP, arachidonate 5-lipoxygenase-activating protein; BMI, body mass index; BP, blood pressure; DMEM, Dulbecco’s modified Eagle’s medium; GRAPHIC, Genetic Regulation of Arterial Pressure of Humans in the Community; LT, leukotriene; MI, myocardial infarction; SNP, single nucleotide polymorphism.

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has been implicated in the pathogenesis of atherosclerosis [3,4]. In this pathway, arachidonate is converted into LTA₄ by the enzyme 5-lipoxygenase, when activated by ALOX5AP (arachidonate 5-lipoxygenase-activating protein). LTA₄ is metabolized further to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase [2]. In response to inflammatory and immune stimuli, LTs are secreted into the extracellular space [5] and subsequently bind to G-protein-coupled cell-surface receptors on their target cells [6–9].

The ALOX5AP gene is located on chromosome 13q12.3 and is 28.9 kb in length, encoding 162 amino acids in 5 exons (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=241; [11]). Using a combination of linkage and association analysis, Helgadottir et al. [12] identified ALOX5AP as a susceptibility gene for MI. A haplotype, HapA, defined by four SNPs (single nucleotide polymorphisms) spanning ALOX5AP, was found to confer an approximate 2-fold greater risk of MI in an Icelandic population [12]. A different four SNP haplotype, HapB, within ALOX5AP was found to confer a similarly greater risk of MI in British Northern European Caucasians, recruited from Leicester and Sheffield [12].

The biological mechanism(s) linking HapA and HapB to the risk of MI has not been elucidated, although Helgadottir et al. [12] reported that, when LTB₄ production from calcium-ionophore-stimulated blood neutrophils was compared in MI cases and healthy controls, male MI cases carrying HapA (n = 10) produced the highest amounts of LTB₄ when compared with controls. Male MI cases without HapA (n = 18) also produced more LTB₄ than controls. Although LTB₄ production was greater in male MI cases carrying HapA than male MI cases without HapA, the difference in LTB₄ production between the two groups was not statistically significant [12]. Therefore whether HapA is indeed associated with increased LTB₄ production and whether HapB, the other disease-associated haplotype, also affects LTB₄ production remains unclear. In the present study, we have therefore examined whether healthy subjects carrying HapA or HapB have increased neutrophil LTB₄ production compared with healthy subjects with neither HapA or HapB (non-A/non-B carriers).

**MATERIALS AND METHODS**

**Subjects**

To identify healthy subjects carrying different variants of the ALOX5AP gene, genotypes were determined in 1268 Northern European Caucasians, from 317 nuclear families, recruited in the GRAPHIC (Genetic Regulation of Arterial Pressure of Humans in the Community) study. The GRAPHIC study is a population-based study of representative nuclear families (both parents aged 40–60 years and two children aged 18–40 years), recruited from general practices in Leicestershire, to investigate the impact of candidate gene polymorphisms on BP (blood pressure) and other cardiovascular traits. Details of recruitment have been described previously [13]. Subjects enrolled in the present study were from the parental generation of the GRAPHIC cohort (results from the offspring generation was used only to help assign haplotypes). None of the subjects recruited for the study reported a history of cardiovascular disease. Subjects with inflammatory conditions, inter-current infection or known malignancy were excluded. Subjects in the three groups, i.e. carriers of at least one copy of HapA (HapA group), carriers of at least one copy of HapB (HapB group) and subjects with neither HapA nor HapB (non-A/non-B group), were age- and sex-matched. All of the subjects enrolled provided written informed consent, and the study was approved by the Leicestershire Research Ethics Committee.

**Genotyping**

To identify HapA carriers, HapB carriers and non-A/non-B carriers, the 1268 subject were genotyped for the seven SNPs that define HapA and HapB (Table 1) using an ABI prism 7900HT Sequence Detection System with SDS v2.1 software (Applied Biosystems). Allelic discrimination was achieved using fluorogenic 5′ nucleic activity TaqMan® MGB probe-based assays [12]. Further details of the assays are available from the authors.

**Measurement of ionophore-stimulated neutrophil LTB₄ production**

Neutrophils were isolated from 12 ml of peripheral venous blood collected in EDTA vacutainers. Subjects were seen in the morning in a fasting state, and blood samples

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**Table 1** Description of the SNPs in the ALOX5AP gene that were genotyped to determine HapA and HapB haplotype status

Ref SNP ID refers to the reference for the SNP in the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&dopt=full_report&list_uids=241; [11]). The decode SNP ID is the original identifier given to the SNP by deCode Genetics and is also available on the NCBI site. The relative position refers to position in base pairs from the start of the first exon.

<table>
<thead>
<tr>
<th>Ref SNP ID</th>
<th>deCODE SNP ID</th>
<th>Gene</th>
<th>Relative position</th>
<th>SNPs defining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs17222814</td>
<td>SG13S25</td>
<td>Promoter</td>
<td>–10189G &gt; A</td>
<td>G</td>
</tr>
<tr>
<td>Rs17216473</td>
<td>SG13S377</td>
<td>Promoter</td>
<td>–5777G &gt; A</td>
<td>A</td>
</tr>
<tr>
<td>Rs10507391</td>
<td>SG13S114</td>
<td>Intron 1</td>
<td>2354T &gt; A</td>
<td>T A</td>
</tr>
<tr>
<td>Rs769874</td>
<td>SG13S38</td>
<td>Intron 3</td>
<td>16699C &gt; A</td>
<td>G</td>
</tr>
<tr>
<td>Rp9551963</td>
<td>SG13S32</td>
<td>Intron 4</td>
<td>22005C &gt; A</td>
<td>A</td>
</tr>
<tr>
<td>Rp9315050</td>
<td>SG13S41</td>
<td>Intron 4</td>
<td>26303A &gt; G</td>
<td>A</td>
</tr>
<tr>
<td>Rs17222842</td>
<td>SG13S35</td>
<td>3′-UTR</td>
<td>30375G &gt; A</td>
<td>G</td>
</tr>
</tbody>
</table>
Leukotriene B$_4$ production in healthy subjects carrying variants of the ALOX5AP gene

RESULTS

Subjects

The flow of subjects through the study is summarized in Figure 1. Of the 634 parental generation subjects genotyped, haplotype phase could not be determined in 63 (10%). Of the remainder, 24.3% subjects carried HapA and 9.3% subjects carried HapB. On the basis of age- and gender-matching, 148 subjects were sent letters inviting them to participate in the study, and 59 subjects were recruited: 21 HapA carriers, 20 HapB carriers and 18 non-A/non-B carriers. The characteristics of the three haplotype groups are shown in Table 2. The three groups were well matched for all variables, apart from a slight difference in mean ambulatory 24-h diastolic BP. All subjects had a normal white blood cell count.

Effect of ALOX5AP haplotype on LTB$_4$ production

LTB$_4$ production in non-stimulated cells was negligible (range, 0.01–0.03 ng/10$^6$ cells). The net stimulated production of LTB$_4$ for subjects in the different haplotype groups are shown in Figure 2. There was no difference in the mean level for LTB$_4$ production in the three groups (non-A/non-B, 24.9 ± 8.3 ng/10$^6$ cells; HapA, 22.2 ± 11.9 ng/10$^6$ cells; HapB, 19.8 ± 4.8 ng/10$^6$, $P = 0.14$). Stimulated LTB$_4$ production in one HapA subject was 68.3 ng/10$^6$ cells and was inconsistent with the rest of the dataset (Figure 2). When this outlier was excluded, the distribution of LTB$_4$ production between the haplotype groups remained non-significant ($P = 0.11$).

There was no association between LTB$_4$ production and age ($P = 0.94$) (although the age range was small), gender ($P = 0.93$), smoking status ($P = 0.10$), BMI ($P = 0.87$) or total cholesterol level ($P = 0.21$).

To assess the variability of the assay, the intra-individual coefficient of variation was measured from the readings of samples obtained from the same subject on three separate occasions, separated by 3 weekly intervals. This gave a coefficient of variation of < 16%.
DISCUSSION

Both descriptive as well as interventional findings suggest an important role for the 5-lipoxygenase pathway in atherosclerosis. 5-Lipoxygenase expression increases with the evolution of atherosclerotic plaques [4]. In subjects undergoing carotid endarterectomy, LTB₄ levels are higher in plaque homogenates from symptomatic compared with asymptomatic subjects, suggesting that the pathway may be involved in promoting plaque instability [17]. Antagonism of LTB₄ receptors in hyperlipidaemic mice reduces lipid accumulation and monocyte infiltration in atherosclerotic lesions when compared with hyperlipidaemic control mice [18]. Finally, genetic knock-out of the 5-lipoxygenase gene in atherosclerosis prone mice produces a marked reduction in atheroma formation [3]. It has been proposed that LTB₄-induced activation of leucocytes leads to release of lysosomal enzymes, such as myeloperoxidase, and the generation of reactive oxygen species that have been associated with propagation and acute complication of atherosclerosis [18].

In this context, the finding by Helgadottir et al. [12], starting from an initially an unbiased genome-wide linkage analysis approach, that specific haplotypes of the gene encoding ALOX5AP, the main regulator of the activity of 5-lipoxygenase, are associated with increased risk of MI has raised considerable interest in the role of this gene in explaining some of the genetic susceptibility to coronary artery disease and MI. HapA was also associated with risk of stroke in the Icelandic cohort studied by Helgadottir et al. [12]. Other studies have subsequently also investigated the association between ALOX5AP variants and cardiovascular diseases. HapA was associated with a 36% greater risk of stroke in a Scottish cohort [19]. In a German case-control study [20], HapA was not itself associated with risk of stroke, but one of the single markers (SG13S114 in Table 1) constituting this haplotype was significantly associated. The authors [20] suggested that differences in allele and haplotype frequencies may explain the discrepant results. In a study in Japanese subjects [21], the frequencies of HapA and HapB were too low to examine for an association, but haplotypes constructed from two other SNPs (A162C and T8733A) did influence risk.

The study by Helgadottir et al. [12] suggested that the carriage of HapA may be associated with a greater
Table 2  Demographics of the subjects studied carrying the different haplotypes of ALOX5AP

<table>
<thead>
<tr>
<th>Variable</th>
<th>HapA</th>
<th>HapB</th>
<th>non-A/non-B</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>20</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>42.9</td>
<td>45.0</td>
<td>55.6</td>
<td>0.71</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.1 ± 4.2</td>
<td>56.1 ± 4.1</td>
<td>56.9 ± 3.4</td>
<td>0.36</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>4.8</td>
<td>5.0</td>
<td>11.1</td>
<td>0.68</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8 ± 4.0</td>
<td>27.0 ± 3.9</td>
<td>27.2 ± 4.5</td>
<td>0.96</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.3 ± 0.9</td>
<td>5.9 ± 1.1</td>
<td>5.4 ± 0.9</td>
<td>0.09</td>
</tr>
<tr>
<td>S.D. values are given for each group.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In the present study, we found no evidence for increased LTB₄ production by blood neutrophils when stimulated with the calcium ionophore A23187 in healthy subjects carrying either HapA or HapB of ALOX5AP compared with non-A/non-B carriers. A strength of our present study is that we investigated healthy subjects where any genuine genotype-specific effects should be easier to detect without the effects of confounding by disease or treatment. Furthermore, our subjects were selected from the general population (Figure 1) making any selection bias unlikely. The protocol used for stimulating the neutrophils was very similar to that of Helgadottir et al. [12], making it unlikely that methodological differences masked any effects of the genotype in our present study. Indeed, in pilot studies, we compared LTB₄ production at three different doses of A23187 (0.5, 1 and 2 μmol/l) and three different incubation periods (10, 20 and 30 min) in a series of subjects and independently established that 1 μmol/l and incubation for 20 min, which are the same conditions used by Helgadottir et al. [12], were the optimal conditions (A. Maznyczka and N. J. Samani, unpublished work). A negative finding raises questions about the power of a study. For a functional study with selection on the basis of genotype, our study is moderately large and indeed larger than the initial study by Helgadottir et al. [12]. A post-hoc power calculation showed that, given the sample sizes, means and S.D.s, the study had 90% power for an α of 0.05 to detect a 32% difference in LTB₄ production between either the HapA or HapB group compared with the non-A/non-B group. Thus the present study had reasonable power to detect an effect likely to be of physiological relevance.

Our findings have a number of implications. They indicate that if either the HapA or HapB haplotype of ALOX5AP genuinely increase cardiovascular risk, then the mechanism is not simply due to a systematically observable effect of the haplotype on LTB₄ production in response to stimulation. The other important implication of our present finding is in relation to the use of ALOX5AP inhibitors as therapeutic targets for treating atherosclerotic diseases [5,22]. Although the study by Hakonarson et al. [22] showed that treatment with DG-031 reduced biomarkers of cardiovascular risk in subjects carrying HapA, our results raise the question as to whether the effect is quantitatively or qualitatively genotype-specific or likely to be observed to a similar degree in all subjects. This could have important bearing on the future clinical utility of this class of drugs and the design of outcome trials.

Neutrophils are a major source of LTs and a convenient cell to study. However, it remains possible that the
ALOX5AP haplotypes affect LT production in other cell types involved in atherosclerosis, perhaps in the vessel wall. Furthermore, it may be interesting to measure other relevant products of the 5-lipoxygenase pathway, such as LTC4 (a potent vasoconstrictor of coronary arteries [23]), or in vivo LT production, by quantifying urinary concentration of LTB4 metabolites and cysteinyl LT metabolites [24] to elucidate whether or not these vary by genotype. Finally, we studied healthy subjects and it is possible that the ALOX5AP haplotypes associated with a risk of MI affect LT production differently in the presence of cardiovascular risk factors or disease. These limitations of our present study need to be borne in mind.

In summary, in stimulated blood neutrophils isolated from healthy subjects, we were unable to show any effect on LTB4 production of two haplotypes of the ALOX5AP gene that have been recently associated with a risk of MI. Although the findings do not exclude the possibility that the haplotypes do confer increased cardiovascular risk through some other mechanism, they suggest that knowledge of a patient’s haplotype may not provide useful information on the likely clinical response to ALOX5AP inhibitors.

ACKNOWLEDGMENTS

We thank the investigators of GRAPHIC for allowing us to analyse DNAs and approach subjects from the study. In particular, we would like to thank Mrs Kim Mason for clerical assistance in contacting subjects. We also thank Mr Glenn Cruse for advice regarding the neutrophil isolation and LT assays, Miss Gail Lavery and Mrs Clare Bodycot for help with genotyping, and Miss Martha James for help with setting up the LT assay. A. M. was supported through a bursary from the Health Foundation and LT assays, Miss Gail Lavery and Mrs Clare Bodycot for help with genotyping, and Miss Martha James for help with setting up the LT assay. A. M. was supported through a bursary from the Health Foundation Chair. Finally, we thank the subjects for volunteering to participate in the project.

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10. Reference deleted

Received 25 September 2006; accepted 18 December 2006

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Published as Immediate Publication 18 December 2006, doi:10.1042/CS20060271