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Full Title: Mechanisms of vascular dysfunction in COPD and effects of a novel soluble epoxide hydrolase inhibitor in smokers

Running head: Vascular dysfunction in COPD and sEH inhibition in smokers

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Abstract

Background: Smoking and chronic obstructive pulmonary disease (COPD) are risk factors for cardiovascular disease, and the pathogenesis may involve endothelial dysfunction. We tested the hypothesis that endothelium-derived epoxyeicosatrienoic acid (EET)-mediated endothelial function is impaired in patients with COPD, and a novel sEH inhibitor GSK2256294 attenuates EET-mediated endothelial dysfunction in human resistance vessels both *in vitro* and *in vivo*.

Methods: Endogenous and stimulated endothelial release of EETs was assessed in 12 COPD patients, 11 overweight smokers, and 2 matched control groups, using forearm plethysmography with intra-arterial infusions of fluconazole, bradykinin, and the combination. The effects of GSK2256294 on EET-mediated vasodilatation in human resistance arteries were assessed *in vitro* and *in vivo* in a Phase 1 clinical trial in healthy overweight smokers.

Results: Compared to controls, there was reduced vasodilatation to bradykinin (p=0.005), blunted effect of fluconazole on bradykinin-induced vasodilatation (p=0.03), and a trend towards reduced basal EET/DHETs ratio in COPD patients (p=0.08). A similar pattern was observed in overweight smokers. *In vitro,* 10 μM GSK2256294 increased 11,12-EET-mediated vasodilatation compared to vehicle (90±4.2% vs. 72.6±6.2% maximal dilatation), and shifted the bradykinin EC50 (-8.33±0.172 vs. -8.10±0.118 logM; p=0.001 for EC50). *In vivo,* 18 mg GSK2256294 improved the maximum bradykinin response from 338±46% pre-dose to 566±110% post single dose (p=0.02), and to 503±123% post chronic dose (p=0.003).

Conclusion: GSK2256294 attenuates smoking related EET-mediated endothelial dysfunction, suggesting potential therapeutic benefits in patients with COPD.

Trial Registration: ClinicalTrials.gov NCT01762774

Key words: soluble epoxide hydrolase inhibitor, endothelial function, smokers, clinical trial, EETs, COPD

Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide, and a risk predictor for atherosclerosis.[1,2,3] Several pathophysiological processes may contribute to disease progression, and increased cardiovascular risk in COPD, including systemic effects of smoking, chronic inflammation,[4] and endothelial dysfunction.[5] Patients with COPD are also more likely to suffer from other cardiovascular co-morbidities, including central abdominal obesity, and the metabolic syndrome, particularly in earlier stages of COPD.[6–8] Endothelium-derived hyperpolarising factors (EDHF), particularly epoxyeicosatrienoic acids (EETs) are involved in the modulation of vascular tone,[9] attenuation of inflammation,[10] and activation of fibrinolysis by augmenting tissue plasminogen activator (tPA) expression.[11]

EETs are synthesised by cytochrome P450 (CYP) enzymes, and metabolised to their less biologically active diols by soluble epoxide hydrolase (sEH) enzymes.[12] Smoking has a synergistic effect with CYP450 and sEH polymorphisms,[13] resulting in enhanced sEH activity, reduced plasma EETs, and increasing overall risk of myocardial infarction.[14] Plasma EETs levels are reduced in patients with coronary artery disease who are obese, or who smoke.[15] EETs are also produced in lung epithelial cells, and they may become dysfunctional in COPD.[12] *In vivo*, smokers exhibit reduced endothelial responses to bradykinin,[5] and this may be associated with impaired EDHF-mediated vasodilatation.[16,17] However, the functional role of EETs has not yet been characterised in humans.

Up-regulation of EETs by sEH inhibition in animals improves the metabolic syndrome,[18] lung function, and attenuates smoking-related inflammation and emphysema.[19] GSK2256294 is a novel potent sEH inhibitor in Phase 1 clinical development, and may have the potential to impact upon systemic and pulmonary endothelial function. As this was a Phase 1 clinical trial, mainly focussed on safety, and tolerability in the healthy, we used a cohort of overweight smokers as a representative for patients with early stage COPD.

We hypothesised that EETs synthesis is reduced in patients with COPD and otherwise healthy overweight smokers, and that sEH inhibition would up-regulate EETs and endothelial dysfunction. We completed a physiological study, in which we assessed EETs-mediated basal tone, and the EETs component of bradykinin stimulated vasodilatation in patients with COPD, and in overweight smokers, in order to maximise the impact of cardiovascular risk factors in otherwise healthy subjects. Subsequently, we examined the effects of a novel sEH inhibitor GSK2256294 in human resistance arteries *in vitro*, and *in vivo* in a Phase 1 clinical trial with an

experimental medicine arm to provide early proof-of-mechanism for target engagement in overweight smokers. The study design, safety and pharmacokinetic data from the Phase 1 trial were reported separately,[20] and we only report the effects of sEH inhibition on endothelial function in this manuscript.

Materials and Methods

All study procedures were conducted in accordance with the Declaration of Helsinki, approved by appropriate institutional review boards, and received favourable opinions from local ethics committees (13/EE/0032, 12/LO/1832), and the Medicines and Healthcare products Regulation Agency. Analysis and Statistical methods are described in the supplementary material. All subjects were recruited following written consent.

We used forearm venous occlusion plethysmography[21] to assess vascular function *in vivo* with intra-arterial infusion of challenge agents via a 27-gauge needle (Coopers Needleworks, Birmingham, UK) inserted into the brachial artery. Venous plasma concentrations of EET/DHETs were assessed as a representative of sEH activity at baseline, and during the forearm blood flow studies. Oscillometric blood pressures were monitored in the non-infused arm. Detailed methods and statistical analyses are in the supplementary material.

Study 1

Twelve male COPD patients (FEV1/FVC < 0.7 and FEV1 < 80% post-bronchodilator), and 12 healthy gender matched controls (matched controls 1) underwent a single forearm blood flow study to assess EETs mediated vasodilatation (UKCRN Portfolio ID: 14339). Patients taking concomitant medications which interfere with CYP450 or cyclo-oxygenase enzymes were asked to stop for at least 4 days prior to the forearm blood flow. Overall endothelium-dependent function was assessed by infusing bradykinin (100, 300 and 1000 pmol/min, Bachem Distribution Services GmbH, Germany), and stimulated EETs release was assessed by co-infusing bradykinin with 0.4 µmol/min fluconazole; a cytochrome P450 inhibitor which inhibits EETs synthesis (Pfizer Ltd, Kent, UK).[9] (**Supplementary material figure 1**). Endothelium-independent responses were assessed using 12, and 38 nmol/min (3, and 10 µg/min) sodium nitroprusside (SNP) (Nitroprussiat FIDES, Madrid, Spain).

Study 2

Twelve overweight smokers (≥10 cigarettes/day, and >5 pack-year history, weight >60kg and BMI 28-35kg/m^2), and equal numbers of healthy gender and age-matched non-smoker controls (matched controls 2) underwent the same forearm blood flow protocol as subjects in Study 1.

Study 3

We first assessed the effects of sEH inhibition, *in vitro,* by application of GSK2256294 to human resistance arteries treated with L-nitroarginine methyl ester (LNAME), and indomethacin (detailed methods in supplementary material), and *in vivo*, using forearm blood flow pre-dose, after a single dose (acute effects), and after 14 days (chronic effects) of oral GSK2256294. Responses to bradykinin (300, 600 and 1000 pmol/min) were assessed in the presence of 8 μmol/min N^G-monomethyl-L-arginine (LNMMA; Bachem) and 6 mmol (1 g) intravenous aspirin (Aspergic Sanofi-Aventis, France) to inhibit NO and PGI_2 syntheses in order to maximise EDHF and EETs (**Supplementary material figure 2**). Venous concentrations of t-PA and plasminogen activator inhibitor-1 (PAI-1) were measured before and after each dose of bradykinin.[22] Challenge agent doses were chosen based on previous studies.[5,5]

To assess the effects of GSK2256294 *in vivo*, we studied healthy overweight smokers (no concomitant medications) as a paradigm for a COPD population in a Phase 1 clinical trial to provide early proof of mechanism (ClinicalTrials.gov NCT01762774). Thirty male overweight smokers, were allocated in a 2:1 ratio between GSK2256294 (6 mg or 18 mg) and placebo for 14 days repeat dose. GSK2256294 doses were chosen based on enzyme inhibition and pharmacokinetic data from the single dosing cohorts.[20]

Results

Study 1

Subject demographics are presented in **Table 1**. The average FEV1 was 53±13% predicted, and FEV1/FVC ratio was 0.5±0.1 in the COPD subjects. There was a trend towards higher plasma concentration of basal EET/DHETs ratio in matched controls 1 compared to COPD patients (0.54±0.12 vs. 0.45±0.14; p=0.08) (**Figure 1**).

There was a dose-dependent increase in forearm blood flow ratio following bradykinin in both groups (p<0.0001). Bradykinin response was significantly higher in matched controls 1 than patients with COPD (maximal dilatation 1314±191% vs. 552±103%; p=0.005) (**Figure 2A**). In the presence of fluconazole, maximum

dilatation to bradykinin was reduced in matched controls 1 (406±64%; p<0.0001), but not in patients with COPD (447±124%; p=0.32), showing a significant between group difference in inhibition (p=0.03). There was no difference in SNP response between groups (data not shown). Blood pressure remained constant throughout the studies.

Although not significant, plasma concentrations of EET/DHETs ratio in response to bradykinin was higher in matched controls 1 compared to patients with COPD (maximum 8.6±3.4 vs. 6.8±1.1; p=0.83). In the presence of fluconazole, although not significant, total EET/DHETs were slightly less in matched controls 1 (maximum 4.7±0.4; p=0.27), but not in patients with COPD (5.2±0.9; p=0.70) (**Figure 3A**).

Study 2

Although not significant, basal EET/DHETs ratio was higher in matched controls 2 compared to overweight smokers (0.46±0.06 vs. 0.39±0.04; p=0.33) (**Figure 1**).

Bradykinin response was higher in matched controls 2 than overweight smokers (maximal dilatation: 930±81% vs. 575±112%; p=0.02) (**Figure 2B**). In the presence of fluconazole, maximum dilatation to bradykinin was reduced in matched controls 2 (400±49%; p<0.0001), but not in overweight smokers (437±57%; p=0.16), resulting in a significant between group difference (p=0.002). There was no difference in SNP response between groups (data not shown). Blood pressure remained constant throughout the studies. There was no difference in the bradykinin response between COPD and overweight smokers (p=0.72).

Although not significant, the increase in EET/DHETs ratio in response to bradykinin was higher in healthy matched controls 2 compared with overweight smokers (maximum 10.31±4.43 vs. 5.66±0.46; p=0.80). In the presence of fluconazole, EET/DHETs were reduced in matched controls 2 but slightly increased in overweight smokers (maximum 5.02±0.38 vs. 8.19±2.18; p=0.003) (**Figure 3B**).

Study 3

In LNAME and indomethacin treated resistance vessels, GSK2256294 10 µM increased 11,12-EET-mediated vasodilatation compared to vehicle (n=6 in each group, 90±4% vs. 73±6% maximal dilatation; **Figure 4A**), and shifted the bradykinin EC50 (n=6, -8.33±0.17 vs. -8.10±0.12 logM; p=0.001; **Figure 4B**). However, vasodilatation to 8,9-EET was unaltered (82±16% vs. 72±19%, maximal dilatation), suggesting that the effects were regio-isomer specific. The vasodilatation to

papaverine (100 μM), a test of direct smooth muscle vasodilatation, was unchanged in GSK2256294.

In vivo, 28 subjects, including the 11 who took part in the physiological study, completed forearm blood flow studies at pre-dosing, after a single dose, and after 14 days repeat dosing with placebo (n=6) or GSK2256294 6 mg or 18 mg (n=11 in each group) (**Table 1**). There was a trend to increased bradykinin response after single and repeat dosing in the active treatment groups. In subjects who received 6 mg, response to bradykinin increased by 23±17% on day 1 and by 22±22% on day 14. In those who received 18 mg, bradykinin response increased by 14±17% on day 1, and 12±14% on day 14. Responses to SNP did not change.

In a *post-hoc* analysis of forearm blood flow ratio, there was an improvement in bradykinin-induced responses following dosing with the active drug compared to placebo (p=0.007), with the greatest effect in the active drug 18 mg group. In the 18 mg active drug group, the maximum bradykinin response improved from 338±46% pre-dose to 566±110% post single dose (p=0.02), and to 503±123% post chronic dose (p=0.003). (**Figure 5**).

LNMMA and aspirin inhibited basal flow equally on all three days in the three treatment arms (**Supplementary material Table 1**). Blood pressure remained stable, and there were no changes to t-PA in response to BK, or in PAI-1 release (data not shown).

Discussion

The findings from these studies suggest that COPD and smoking are associated with impaired overall endothelial function, and reduced stimulated vascular EETs production. Proof-of-mechanism data demonstrates that sEH inhibition with GSK2256294 results in improvements in vascular function both *in vitro* and *in vivo*.

We elected to study patients with COPD and overweight smokers as the mechanisms behind COPD, smoking and cardiovascular disease remains poorly understood. Both smokers and patients with COPD exhibit low-grade systemic inflammation,[1] which plays a key role in endothelial activation, resulting in endothelial dysfunction, and the initiation of atherosclerosis.[23] It has been demonstrated that patients with COPD,[5] smokers,[24] and ex-smokers[25] exhibit a similar degree of endothelial dysfunction, suggesting that smoking may be the key contributing factor. Cardiovascular risk factors are more likely to cluster in obesity, manifesting as a syndrome of increased adipocytes, hyperglycaemia, and dyslipidaemia, with

underlying low-grade inflammation. In normotensive overweight subjects with the metabolic syndrome, acetylcholine-, rather than bradykinin-induced vasodilatation is reduced, possibly suggesting a lesser degree of endothelial dysfunction.[9] However, the extent to which EETs contributed to this endothelial dysfunction remained unclear, and our study was the first to interrogate this further, and forearm blood flow data suggest that EETs production is similarly impaired in patients with COPD and overweight smokers, supported by plasma quantification of EETs/DHETs as a representative of sEH activity.

We observed a trend towards reduced baseline EET/DHETs in patients with COPD and overweight smokers, and when comparing the two matched control groups, baseline EET/DHETs ratio are slightly less in the younger matched controls for overweight smokers (matched control 2), than those for COPD (matched control 1). However, human plasma EETs, and DHETs are notoriously difficult to quantify, due to their instability, thus, definitive conclusions cannot be drawn from these insignificant results, but can only be taken in context of our forearm blood flow data, and previous published data. In animals, obesity is associated with reduced hepatic expression of EETs-producing CYP2C enzymes.[26] In mesenteric arteries of obese Zucker rats, there is reduced CYP2C and CYP2J enzymes, with enhanced activity of sEH enzymes.[27] Increased sEH activity may represent more advanced inflammation, as in coronary artery disease, those who are obese or who smoke exhibit the lowest EET/DHETs ratio.[15] sEH activity is associated with forearm blood flow, as subjects with Lys55Arg polymorphism in the sEH encoding gene (*EPHX2*) exhibit higher sEH activity, and reduced vasodilator responses to bradykinin.[28] Smoking can also significantly upregulate *EPHX2*[29], and this is associated with increased coronary artery calcification in man.[13]

The reduced EETs synthesis and endothelial dysfunction observed in patients with COPD and overweight smokers, may be a result of chronic low grade inflammation secondary to smoking.[30] In animals, dimethylsulfoxide-soluble smoke particles can up-regulate endothelium-derived vasoconstrictors via the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB);[31] a pivotal protein controlling the transcription of genes relevant to the pathophysiology of the blood vessel wall, including adhesion molecules and cytokines.[32] EETs exert their anti-inflammatory effects by inhibiting the activation of NFκB.[32] Inflammatory states are associated with down-regulation of hepatic and extrahepatic CYP450 enzymes, resulting in a vicious cycle of reduced EETs production and an ineffective EETs-mediated anti-inflammatory effect both locally and systemically.[33]

GSK2256294 is a potent sEH inhibitor, which exerts high levels of sEH enzyme inhibition both *in vitro*,[19] and *in vivo.*[20] In human left internal mammary arteries, 11,12-EETs are the most potent regio-isomer,[34] and we confirmed that both 11,12-EETs and bradykinin mediated vasodilatation were enhanced in the presence of GSK2256294 in human resistance arteries. In animal models of cigarette smoking and obesity, sEH inhibition improves lung,[35] and endothelial function,[36] and attenuates pulmonary inflammation as reflected by reduced inflammatory cells including neutrophils, and macrophages.[19] In human bronchial cells, treatment with exogenous EETs protects against cigarette smoke extract–induced injury.[37] Consistent with *in vitro* results, both acute and chronic sEH inhibition for up to two weeks improve responses to bradykinin.

No changes were observed in t-PA release following sEH inhibition. t-PA is a fibrinolytic serine protease that is released from the endothelium, and regulates degradation of intravascular fibrin. Impaired t-PA release can be associated with coronary atherosclerosis, and cigarette smoking.[25] Treatment of human endothelial cells with exogenous EETs, particularly 11,12-EETs, can increase t-PA protein expression in a dose and time-dependent manner, possibly due to activation of a G-protein, whilst not affecting plasminogen activator inhibitor type 1 (PAI-1), the endogenous inhibitor of t-PA.[11] t-PA release may also be dependent on the agonist, and in this group of overweight smokers, substance P may elicit a greater response.[24]

Some limitations of this study warrant consideration. As the main focus of the Phase 1 clinical trial was on safety, tolerability, and pharmacokinetics of GSK2256294 in healthy volunteers, we were not able to test this novel drug in COPD patients. In addition, the lack of non-smoking controls in the Phase 1 clinical trial means that the magnitudes of the effects of both doses of GSK2256294 were relatively small, and similar to the variance in bradykinin responses in the placebo group. Therefore, Phase 2 studies in larger patient groups are required to draw definitive conclusions.

Some evidence also suggests that in NO deficient conditions, EETs may be upregulated.[9] Thus, by creating an NO deficient milieu during the forearm blood flow study with LNMMA, we may have masked any further up-regulation of EETs by sEH inhibition. Larger clinical trials in patients with COPD, without concomitant inhibition of nitric oxide synthase, would be required to further understand the clinical impact of sEH inhibition. This must also be approached with caution for the potential of EETs to stimulate angiogenesis, and possibly modulate cancer genesis and metastases,[38] though, interestingly, dual action cyclo-oxygenase and sEH inhibition may in fact

suppress cancer.[39] We found no changes in serum vascular endothelial growth factor the active drug group with this dosing regime after 14 days.[20]

Conclusions

Patients with COPD and overweight smokers have impaired endothelial function, and dysregulated EETs signalling. sEH inhibition can augment bradykinin-induced vasodilatation in human resistance vessels both *in vitro* and *in vivo,* suggesting that sEH inhibition may be a novel therapeutic target to ameliorate cardiovascular risk in patients with smoking related endothelial dysfunction.

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