Cigarette smoke enhances chemotaxis via acetylation of proline-glycine-proline

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1. ABSTRACT

Several chronic lung diseases have been linked to cigarette smoking (Chronic Obstructive Pulmonary Disease (COPD), and cancer are associated with increased tobacco use). We recently described a collagen fragment, proline-glycine-proline (PGP), chemotactic for neutrophils, that appears to play a role in COPD, cystic fibrosis, and bronchiolitis obliterans syndrome. PGP can exist in either its native or acetylated form (NAcPGP), although the mechanism of N-terminal-acetylation remains unknown. This work investigates the possibility that cigarette smoke (CS) and its components acetylate PGP, describing a possible mechanism for some of the chronic inflammation seen in tobacco-associated disease. CSE and CSC (3.56 and 12.38 ng/ml NAcPGP respectively, p less than 0.01) and its components (acrolein, acetaldehyde, and methyl glyoxal) acetylated PGP (0.51, 1.03, and 0.23 ng/ml NAcPGP, p less than 0.01). Both N-acetyl-cysteine and carbocysteine (scavengers of reactive aldehydes) blocked chemical acetylation of PGP by CS (100% and 97% inhibition, respectively, p less than 0.01). NAcPGP is more chemoattractive to neutrophils, and less susceptible to degradation by Leukotriene-A₄-Hydrolase (detected in the lung). These experiments propose a mechanism for the increased neutrophil recruitment seen in smoking-associated lung diseases.

2. INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are an integral part of the innate immune system. PMNs are recruited to sites of inflammation to resolve infections, in part, through release of their potent proteases. (1) However, chronic PMN inflammation has been implicated in the progressive destruction/obliteration of the airways in several pulmonary diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and bronchiolitis obliterans syndrome (BOS). (2-5) Proline-Glycine-Proline (PGP) and its N-acetylated form (NAcPGP) are recently described neutrophil chemoattractants formed from the breakdown of extracellular matrix collagen in response to inflammatory stimuli. (6) PGP and NAcPGP have been shown to be elevated in COPD, CF, and BOS. (7, 8) Additionally, PGP and NAcPGP act via CXCR1 & 2 receptors, similarly to other neutrophil chemokines (i.e. interleukin 8 (IL-8)). (6) The breakdown of collagen into PGP is accomplished via a multi-step process. Collagen is initially digested into fragments 30-100aa in length by matrix metalloproteases (MMPs) 8 and/or 9. These oligomers are further cleaved into PGP by prolyl endopeptidase (PE). (7) The additional action of one or several as yet unidentified acetylating agents is required to modify PGP to the more active NAcPGP.
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NAcPGP has been shown to be 4-7 times more potently chemotactic to neutrophils than its non-acetylated counterpart and has been implicated in chronic inflammatory diseases. (4) We recently demonstrated that Leukotriene A₄ Hydrolase (LTA₄H) aminopeptidase activity limits acute inflammation by degrading PGP, but N-terminal acetylation of the tri-peptide prevents such degradation. (9) Thus acetylation confers both potency and longevity to the peptide, dramatically increasing its bioavailability and effectiveness.

To date, there have been no observations of an enzyme capable of performing N-terminal acetylation of a proline. While it is possible a proline acetylating enzyme may yet be discovered, non-enzymatic (chemical) acetylation of PGP is a distinct possibility. One logical source of compounds capable of chemical acetylation of PGP is cigarette smoke (CS) and CS derivatives (extract, condensate, and vapor). CS is a known airway irritant involved in inflammatory processes. (10) It is well established that smoking is the primary risk factor for the development of COPD, a disease strongly associated with airway remodeling, elevated PMN burden, and increased levels of both PGP and NAcPGP. (8, 11, 12) Furthermore, cigarette smoke contains volatile acetyl containing compounds such as acetaldehyde and acrolein, each of which we hypothesize should be able to perform the necessary reaction to form NAcPGP. (13)

3. MATERIALS AND METHODS

3.1. Materials

DMSO was purchased from Sigma-Aldrich (St. Louis, MO). 3µM transwell chemotaxis plates were purchased from Millipore (Billerica, MA). 3R4F standardized research cigarettes were obtained from the University of Kentucky (Lexington, KY). Acrolein was obtained from Alfa Aesar (Ward Hill, MA). Acetaldehyde was from Sigma-Aldrich, Glyoxal from Fisher Scientific (Pittsburgh, PA), and Methyl Glyoxal from MP Biomedicals.

3.2. Methods

3.2.1. Preparation of cigarette smoke extract or condensate (CSE or CSC)

Using a three-way stopcock to control flow, a lit standard research cigarette (University of Kentucky, Lexington, KY) was connected to an empty 10ml syringe and a 60ml syringe containing a known volume of PBS (CSE) or DMSO (CSC) (1ml/cigarette). 10ml of smoke were drawn from the cigarette over a 2 second period, and the smoke was bubbled up through either solution over a 10-second push. This was done at a rate of three puffs/minute. The resulting extract was measured for absorbance at 320nm on a spectrophotometer and diluted to the appropriate optical density in PBS or DMSO for all experiments.

3.2.2. CSE/CSC PGP acetylation assay

A single concentration of PGP (100ng/mL) in PBS was combined with varying concentrations of CSE/CSC (0x, 1x, 5x, and 10x where 1x is equal to a 0.1 optical density at 320nm) into Eppendorf tubes at a final volume of 200µL. Samples were incubated at 37°C for varying time points. After completion, the samples were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for PGP and NAcPGP concentrations.

3.2.3. CSE/CSC vapor acetylation assay

100x CSE or CSC was loaded into 6 wells of a 96-well microtiter plate. PGP (100ng/ml) was loaded into 6 wells on the opposite side of the plate with only the plastic cover placed over the plate to allow for vapor transfer, the plate was the incubated at 37°C for 24h. As a control, a duplicate plate was sealed to prevent any vapor effects.

3.2.4. PMN chemotaxis assay

Increasing concentrations of CSE or CSC were preincubated with PGP for 1h at 37°C prior to inclusion in a chemotaxis assay. PGP (100µg/ml) or PGP+CSE/CSC was placed in the bottom well of a multi-leveled plate at 37°C for 1 hour with 2 x 10⁶ PMN layered in the top well. After 1 hr the top plate was removed and cells in the bottom well were counted using an LSR II Flow Cytometer. (4) The addition of both CSE and CSC significantly increased the chemotactic potential of PGP compared to media control (* p<0.001 to media, † p<.05 to PGP alone).

3.2.5. CSE/CSC PGP acetylation assay with N-acetyl-cysteine and carbocysteine

A single concentration of PGP (100ng/mL) was combined in an Eppendorf tube with 10mM of either N-acetyl-cysteine (NAC) or carbocysteine. CSC or CSE (10x) was added to the mixture for a final volume of 1ml. Samples were incubated at 37°C for varying time points. After completion, the samples were again analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for PGP and NAcPGP concentrations.

3.2.6. CSC/CSE vapor acetylation assay with NAC and carbocysteine

High dose CSC or CSE (100x) at a volume of 200µl was loaded into a 96-well microtiter plate. PGP (~100ng/mL) alone, PGP combined with NAC (10mM), or PGP combined with carbocysteine (10mM) were loaded into wells on the opposite side of each plate. As before, a plastic cover was placed over the plate, and sample was then incubated at 37°C for 24 hours. The samples were analyzed using ESI-LCMS/MS for PGP and NAcPGP concentrations.

3.2.7. Statistical analysis

Statistical analysis was performed using a Student’s T Test, all experiments were performed multiple times with at least an “n” of 3.

4. RESULTS

4.1. Cigarette smoke extract (CSE), condensate (CSC), and cigarette smoke constituents acetylate PGP

Cigarette smoke extract (CSE) and cigarette smoke condensate (CSC) were prepared by dissolving the
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To evaluate the potential for volatile effects of CSE and CSC, 100x concentrations of both were placed in the last column of wells of a 96-well microtiter plate with an identical volume of PGP loaded into the first column. The samples were incubated at 37°C overnight and PGP was analyzed for N-terminal acetylation. While not at the same levels seen with direct co-incubation, there was a measurable and significant increase in acetylation compared to controls (plates were sealed to prevent vapor transfer) (Figure 1B, p<0.01). This was particularly interesting in that it indicated a volatile component of CSE/CSC was capable of performing the proline acetylation. Upon conducting a literature search of the known volatile compounds in cigarette smoke, we determined that acrolein, acetaldehyde, glyoxal, and methyl glyoxal could potentially perform the necessary chemical reaction.

We performed a similar assay to the previous experiments using increasing doses of acrolein, acetaldehyde, glyoxal and methyl glyoxal with PGP. Samples were incubated at 37°C and NAcPGP concentration was determined with mass spectrometry. Acrolein, acetaldehyde, and methyl glyoxal all dose-dependently acetylated PGP but there was no measureable PGP acetylation by glyoxal, enabling the compound to function as a negative control (Figure 1C, p<0.01).

4.2. Cigarette smoke acetylation by CSE and CSC increase PGP mediated neutrophil chemotaxis

Using an established chemotaxis assay, we sought to determine if the N-terminal acetylation of PGP was capable of causing a biological effect (4). Indeed, when pre-incubated with CSE and CSC, PGP demonstrated a significant increase in the ability to cause neutrophil migration compared to both PGP and media controls (Figure 2, p<0.01). This effectively demonstrates that chemical modification of PGP is not merely an interesting phenomenon, but can also have an impact in physiologic settings.

4.3. N-acetyl-cysteine and carbocysteine prevent CSE and CSC acetylation

N-acetyl-Cysteine (NAC) and carbocysteine are mucolytic antioxidant agents that are known scavengers of reactive aldehyde species. They are currently prescribed as mucolytics for COPD but appear to have additional therapeutic effects. (14, 15) CSC or CSE was incubated with PGP in the presence of NAC or carbocysteine for various time periods and analyzed for PGP acetylation. NAC and carbocysteine effectively inhibited acetylation of PGP by CSE and CSC compared to controls without either mucolytic agent (Fig 3A, p<0.01). The PGP vapor acetylation assay was repeated with the addition of NAC and carbocysteine, and similar to the direct acetylation experiment, minimal acetylation of PGP occurred in samples with either mucolytic/scavenging agent compared to samples without NAC or carbocysteine. This was observed with both CSC and CSE (Figure 3B, p<0.01). These data support a possible mechanism whereby reducing compounds such as NAC and carbocysteine may diminish lung inflammation in COPD and other chronic lung diseases by inhibiting PGP acetylation, thereby facilitating PGP degradation by LTA4H.

CS and its components are known to have long-term pulmonary consequences, including leading to COPD and lung cancer. (2) The body of enzymes known to be involved in acetylation reactions is growing, yet to our knowledge, none of these enzymes can directly acetylate the imine position of a proline residue. However, there are known reactions (oxidation, glycosylation, acetylation, etc.) that can directly modify proteins in the lungs. (16, 17) Thus, we searched for possible chemical means, focusing on CS initially due to the high amount of NAcPGP seen in a mouse model of COPD and human COPD patients. (8, 18) To our knowledge, there has been no prior report of direct chemical acetylation of proteins by CS, nor has there been a demonstrated effect on the modification of a small peptide with biological consequences.

5. DISCUSSION

In this work, we demonstrate that CSE, CSC (Figure 1A) and CSE/CSC vapor (Figure 1B) are capable of acetylating the chemoattractant/matrikine PGP. The dose-dependant increase in PGP acetylation, seen with CSE, CSC, and reactive aldehyde components of CS (acrolein, acetaldehyde, and methyl glyoxal) indicate a functional modification of a matrikine associated with several chronic inflammatory lung diseases. A single cigarette can yield near-milligram amounts of acrolein and acetaldehyde, both of which are retained in the tissues of the lung at high concentrations. (19, 20) Reactive aldehydes are known to possess half-lives ranging from a few hours to days and are capable of diffusing significant distances. (21, 22) Based upon previous work done with PGP and recently published studies, this modification is both proteolytically protective, and instrumental in increasing the activity of the tri-peptide.

To demonstrate that reactive aldehydes are among the components in CS responsible for the acetylation of PGP, we used known aldehyde scavengers, N-Acetyl-Cysteine (NAC) and carbocysteine to inhibit the reaction. (23) Both of these mucolytic agents effectively ablated the chemical reaction of the aldehydes with PGP. What is most intriguing is that both of these agents have been approved for, and are currently in use in humans. The potential for adapting these therapies for COPD is an exciting concept for the future treatment of chronic obstructive lung disease.

Indeed, to confirm that the modification we observed was functional, we performed an ex vivo assay
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Figure 1. (A) CSE or CSC were prepared by bubbling one cigarette/ml through PBS or DMSO respectively, and standardized by defining 1x CSE/CSC as having an OD_{320} = 1.0. CSE or CSC were incubated at varying concentrations and time points with 100ng/ml PGP in PBS at 37°C, 5% CO₂ then analyzed by ESI-LC-MS/MS. CSE and CSC both dose, and time-dependently acetylated PGP (*p<0.01 to control). (B) An assay similar to A was performed with CSE or CSC (both at 100x) placed in separate well of a 96 well plate to test the possibility of vapor (volatile components) acetylation of PGP. CSE and CSC vapor were both capable of PGP acetylation. (C) Aldehyde components of cigarette smoke, acrolein, acetaldehyde, methyl glyoxal, and glyoxal were incubated at various concentrations with PGP (100ng/ml) for 24h at 37°C, 5% CO₂ then analyzed by ESI-LC-MS/MS. Glyoxal does not appear to acetylate PGP, functioning as a negative control. Acrolein, acetaldehyde, and methyl glyoxal all significantly and dose dependently acetylate PGP (*p<0.05).
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utilizing isolated human peripheral blood neutrophils in a chemotaxis assay. PGP, when pre-incubated with CSE and CSC, causes increased neutrophil chemotaxis, similar to the level of cellular migration observed with IL-8. These data indicate that the modification of PGP is not merely of chemical interest, but causes a change in the functionality of the peptide.

This study delineates a pathway for acetylation of a recently described bio-marker and modulator of multiple chronic lung diseases. It is important to note that cigarette smoke is not the only source of reactive aldehydes; indeed, there are physiologic reactions where these products can occur. The potential for endogenous production of both acrolein and acetaldehyde is of high interest in lung pathology. Endogenous acrolein is produced by lipid peroxidation via metal catalyzed oxidation of arachidonate from circulating polyamines through the action of serum amine oxidase on spermine. (24) This can occur either from the metabolism of certain drugs and/or at sites of inflammation through the oxidation of threonine by myeloperoxidase (MPO) to 2-hydroxypropionaldehyde followed by dehydration, yielding acrolein. (24) Normally, acrolein is metabolized by conjugation to glutathione through attack of the sulfhydryl group to the α,β double bond of acrolein. The acrolein-glutathione adduct is further metabolized to 3-hydroxypropylmercaptopuric acid, which is can measured in urine. While it is less likely that the majority of individuals with CF smoke, the endogenous production of acrolein and acetaldehyde in CF, along with smoking’s association with COPD (representing a large patient cohort) makes investigating this pathway worthwhile.

An important feature of this research to highlight is the potential for future discovery of compounds in cigarette smoke that are capable of catalyzing the acetylation reaction, or other components of CS that can directly acetylate an N-terminal proline. Additionally, a clinical trial investigating the effect of carbocysteine and/or NAC treatment on clinical outcomes; exacerbations, lung function, and concomitantly incorporating data on neutrophil burden and concentrations of the biomarkers PGP and NAcPGP in COPD and CF is a logical extension of this research. Finally, this work also raises the question of the possible role cigarette smoke and its constituent(s) play(s) on protein acetylation, a recently described regulatory component of cellular metabolism. (25)

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Figure 3. (A) The mucolytic agents, N-Acetyl-cysteine (NAC) and carbocysteine, were added to wells of a 96 well microtiter plate containing 100 µl of PGP (100 ng/ml) with or without 10x CSE or CSC. Both mucolytic compounds potently inhibited PGP acetylation by the two different cigarette smoke derivatives (* p< 0.01, † p< 0.001). (B) An assay similar to the one described above was performed; however, CSE and CSC were placed in wells at the opposite end of the microtiter plate, with no direct contact between the smoke derivatives and the tripeptide PGP. NAC and carbocysteine were again capable of potently inhibiting the chemical modulation of PGP by CSE (100x) and CSC (100x) vapor (* p< 0.01, † p< 0.001).

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7. REFERENCES


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**Abbreviations:** CF: cystic fibrosis, COPD: chronic obstructive pulmonary disease, PGP: proline-glycine-proline, PE: prolyl endopeptidase, CSE: cigarette smoke extract, CSC: cigarette smoke condensate

**Key Words:** PGP, Neutrophils, Acetylation, Cigarette Smoke, Acetaldehyde, Acrolein

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