Aldehydes in Cigarette Smoke React with the Lipid Peroxidation Product Malonaldehyde to Form Fluorescent Protein Adducts on Lysines

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Cigarette smoke is a risk factor for the development of several diseases, but the exact mechanism responsible has not been well-characterized. Because modification, or adducting, of biomolecules is thought to mediate the toxic effects observed from exposure to a wide variety of harmful chemicals, this study investigated the ability of cigarette smoke to produce specific adducts on a peptide to gain insight into the likely effect on cellular proteins. We describe the modification of the ϵ-amino group of lysine contained in a test peptide with stable fluorescent adducts derived from monofunctional aldehydes occurring in cigarette smoke and malonaldehyde, a product of lipid peroxidation. Utilizing high-performance liquid chromatography, fluorescent measurements, and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, the 1,4-dihydropyridine-3,5-dicarbaldehyde and 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivatives of lysine were identified as products of exposure to cigarette smoke extract and malonaldehyde. These data suggest that cigarette smoke may promote the modification of proteins, like those associated with oxidized low-density lipoprotein, and may contribute to smoking-related disease.

Introduction

Cigarette smoke has been identified as a major risk factor for the development of numerous disease states including chronic obstructive pulmonary disease, both emphysema and chronic bronchitis, asthma and other lung diseases (*1*, *2*), atherosclerosis, and numerous cardiovascular dysfunctions (*3*−*6*). Unfortunately, the mechanism of cigarette smoke-mediated disturbance has not been fully characterized, but it is thought to involve the absorption of tobacco smoke components that then adversely affect cellular function. In particular, the effect of tobacco smoke components on endothelial cell and macrophage function has received much attention (*6*−*13*), but the exact mediator (or mediators) of smoke-associated disease has not been identified.

With regard to vascular disease, the oxidation of low-density lipoprotein (LDL) is known to result in an increased uptake of LDL by macrophages located at the vascular wall, which leads to the transformation of macrophages into foam cells prior to the development of atherosclerotic lesions and plaque (*14*−*16*). The chemical modifications that lead to recognition of modified LDL by macrophage scavenger receptors are thought to involve the derivatization of lysine residues by adducts that increase the overall negative charge of a protein (*17*−*20*). The identification of the specific LDL modification responsible for increased macrophage uptake has been the subject of intense study, and the identification of a fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDHDC)[1] adduct has been identified as one possible product of LDL oxidation (*21*, *22*).

Interestingly, a family of 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde (DHDC) adducts can be generated on amines when exposed to several of the monofunctional aldehydes commonly found in cigarette smoke and the lipid peroxidation product malonaldehyde (MDA) (*23*). These modifications have been extensively characterized on small molecules such as methylamine, but the derivatization of ϵ-amino groups of lysine with these types of adducts on proteins or peptides by cigarette smoke has not been described. Because cigarette smoke contains numerous aldehydes and is known to induce pro-oxidative processes in the lung (*24*−*27*), the purpose of this report was to evaluate the formation of 1,4-dihydropyridine-3,5-dicabaldehydes on a peptide to model protein modifications, which would likely occur in the lung following exposure to cigarette smoke.

Materials and Methods

Materials. Formaldehyde (FA) (37%), acetaldehyde (AA), propionaldehyde (PA), butyraldehyde (BA), and benzaldehyde (ZA) were purchased from Sigma (St. Louis, MO). MDA was

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[1] Abbreviations: DHDC, 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde; HDHDC, 1,4-dihydropyridine-3,5-dicarbaldehyde; MDHDC, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde; EDHDC, 4-ethyl-1,4-dihydropyridine-3,5-dicarbaldehyde; PDHDC, 4-propyl-1,4-dihydropyridine-3,5-dicarbaldehyde; ZDHDC, 4-benzyl-1,4-dihydropyridine-3,5-dicarbaldehyde; CHCA, α-cyano-4-hydroxycinnamic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

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synthesized from MDA bis-(dimethyl acetal) by acid hydrolysis followed by acetone (ACT) precipitation of the sodium salt as described (*28*). Sodium MDA was assayed for purity by reaction with 2-thiobarbituric acid utilizing fluorescent detection (515/553) of HPLC-separated products. MDA bis-(dimethyl acetal) (i.e., tetramethoxy-propane) was used for construction of a standard curve for MDA quatification (*29*, *30*). Unless noted, all other reagents were purchased from Sigma and of the highest purity available.

Peptide Synthesis. The peptide used for all experiments contains a single reactive amine located at the ϵ-amino group of lysine. It is based on the N-terminal acetylated version of residues 28−37 of the calcitonin gene-related peptide [Ac-h-α-CGRP(28−37)] and was synthesized by the Omaha Veterans Administration Peptide Core Facility. The primary structure of the acetylated peptide is as follows: Ac-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2. A 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy) valeric acid linker attached to a polyethylene graft polystyrene support (PAL-PEG-PS) for solid phase peptide synthesis was purchased from Applied Biosystems (Foster City, CA). O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and protected amino acid derivatives were purchased from Chem-Impex International (Wood Dale, IL). Dimethylformamide (DMF), dichloromethane (DCM), acetic anhydride, piperidine, trifluoroacetic acid (TFA), acetonitrile, and diethyl ether were purchased from Fisher Scientific (Pittsburgh, PA). Thioanisole, anisole, and ethanedithiol were purchased from Aldrich (St. Louis, MO).

Ac-h-α-CGRP(28−37) was assembled by continuous flow solid phase methodology on the PAL-PEG-PS support at a 0.5 mmol scale using a Pioneer Peptide Synthesizer (Foster City, CA). α-Amino groups were protected with a fluorenylmethoxycarbonyl (Fmoc) group, and side chains were protected with a trityl group for Asn, a *tert*-butyl group for Ser and Thr, and a *tert*-butyloxycarbonyl group for Lys. After removal of the Fmoc group from the resin with 20% piperidine/DMF (v/v), HBTU- and HOBt-activated Fmoc-Phe-OH in DMF were coupled to the resin. This process was repeated with each amino acid derivative. After assembly, the peptide resin was washed with diethyl ether and dried under vacuum. After it was dried, the N terminus was acetylated by acetic anhydride in DMF. After it reacted for 30 min, the peptide resin was filtered, washed with DCM, and dried under vacuum. Cleavage of the peptide from the resin and removal of the side chain protecting groups was accomplished in a 10 mL mixture of TFA, thioanisole, ethanedithiol, and anisole (9.0/0.5/0.3/0.2, v/v). After it was stirred for 2 h at room temperature, the peptide was precipitated by the addition of cold diethyl ether. The peptide/resin was filtered. The peptide was dissolved in TFA, reprecipitated in cold diethyl ether, and isolated by filtration and lyophilized.

Purification of Ac-h-α-CGRP(28−37) was accomplished by reverse phase-HPLC on a Waters (Milford, MA) model 600 HPLC system. The crude peptide was dissolved in 90% solvent A (0.1% TFA/water) and 10% solvent B (0.095% TFA/acetonitrile) and subjected to a gradient of 10−22% B over 50 min on a semipreparative Vydac (Hesperia, CA) C18 column (10 mm × 250 mm). The flow rate was 4 mL/min, and the peptide was detected by UV absorbance at 230 nm. Fractions containing the pure peptide were collected and lyophilized. The final product coeluted on HPLC with a known sample, and its expected mass was verified by electrospray mass spectrometry.

Cigarette Smoke Extract (CSE) Preparation. A concentrated extract of cigarette smoke in phosphate-buffered saline (PBS) was prepared fresh the day of each experiment (*31*, *32*). Research cigarettes were obtained from the University of Kentucky, Tobacco Health Research division (filtered, code 2R4F). In brief, a cigarette was connected to a vacuum driven pump apparatus designed to simulate the act of smoking, and the mainstream smoke was bubbled through PBS warmed to 37 °C with one cigarette used per 1 mL of volume. Before use, the extract was filtered through a 22 μm filter.

Reaction of Aldehydes with Peptide. Monofunctional aldehyde (FA, AA, PA, BA, or ZA), MDA, and the test peptide were combined at concentrations of 1, 2, and 0.1 mM, respectively, in a final volume of 0.2 mL of PBS at pH 7.4, and the reaction mixture was incubated at 37 °C for 72 h before characterization of the products. When indicated, the monofunctional aldehyde was replaced with CSE, or MDA was omitted to assess modifications under MDA-free conditions. Analytical scale preparation of the modified peptides was conducted with 5 mM monofuctional aldehyde, 10 mM MDA, and 1 mM peptide in PBS, pH 4.5, to improve the yield of desired fluorescent product.

Synthesis of DHDC on Methylamine. Reference molecules containing DHDC adducts on methylamine were synthesized as described by Kikugawa et al. (*23*). In brief, 10 mM methylamine was combined with an equal molar concentration of monofunctional aldehyde and a 2-fold excess of MDA in PBS at pH 7.4. The reaction was incubated for 72 h at 37 °C followed by extraction with chloroform. The modified methylamine was then twice crystallized from the organic solvent with *n*-hexane. The presence of a single unique fluorescent product was verified by TLC utilizing silica gel plates with methanol:chloroform (9:1) as the mobile phase.

Fluorescent Spectra. Further characterization of each modification was conducted by measuring the absorbance to fluorescent emission properties of each adducted peptide sample and comparing the values to identical modifications of methylamine. The maximum fluorescent absorbance (250−650 nm) to emission (425−700 nm) spectra for each sample in water were determined with a Perkin-Elmer LS50B Luminescence Spectrometer.

HPLC Separation of Modified Peptides. HPLC separation of adducted peptides utilized the Beckman System Gold equipment with the 126 Solvent Module, 166 Programmable Detector Module, and the Nouveau software for module control, data collection, and analysis. A Shimadzu RF-530 Fluorescent HPLC Monitor was connected in series for fluorescent data collection. Samples were simultaneously monitored for absorbance at 214 nm (peptide bond) and fluorescence with excitation at 400 nm and emission at 465 nm (dihydropyridine dicarbaldehyde derivatives). An Alltech Platinum, C18 (300A, 5 μ), reverse phase column (250 mm × 4.6 mm) was used for all analysis. Solvent A was composed of water containing 0.1% TFA, and solvent B was composed of pure acetonitrile containing 0.1% TFA. Samples were injected into the column with a flow rate of 1 mL/min and a linear gradient of 15−30% solvent B over 54 min.

HPLC Purification of Fluorescent Peptide Products. Peptides adducted with each of the described modifications were purified at an analytical scale utilizing HPLC equipment identical to that described above. However, the elution gradient was modified as follows: (i) the column was equilibrated with 15% B; (ii) the sample was injected, and solvent B was linearly increased from 15 to 23% over 10 min; and (iii) solvent B was maintained at 23% until the fluorescent peaks eluted from the column. Fractions were collected (0.25 mL) as the peaks of interest eluted from the column. Each fraction was tested for purity by HPLC as described above for the presence of a single detectable peak. Pure fractions were pooled, frozen, and concentrated by lyophilization, and the mass of the purified sample was verified by mass spectroscopy as described below.

Mass Spectroscopy. The mass of the peptide−aldehyde reaction products was assessed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy utilizing the chip-mediated system manufactured by Ciphergen Biosystems, which is also described as surface-enhanced laser desorption/ionization. In brief, the sample (1−3 μL) was spotted onto a H4 hydrophobic interaction chip and allowed to air dry. An energy-absorbing molecule (0.7 μL), composed of saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile: 1% trifluoroacetic acid, was then placed onto the dried sample. The chip was then air-dried followed by data collection using

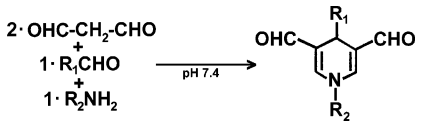

Figure 1. Summary of the reaction of MDA (OHC−CH_2−CHO) and a monofunctional aldehyde (R_1CHO) with a molecule containing a primary amine (R_2NH_2).

Table 1. Expected Physical Characteristics of DHDC Peptide Adducts

MDA	monoaldehyde	R_1 group	adduct	Δ mass	peptide mass
−	none	NA	NA	+0	1060
+	FA	H-	HDHDC	+120	1180
+	AA	CH_3−	MDHDC	+134	1194
+	PA	CH_3CH_2−	EDHDC	+148	1208
+	BA	$CH_3(CH_2)_2$−	PDHDC	+162	1222
+	ZA	C_6H_5−	ZDHDC	+196	1256

the PBSII chip reader. Mass spectroscopy data were collected with the instrument in positive mode, with a laser intensity of 205, time lag focusing optimized for 1500 Da (delayed extraction with a lag of 277 ns), and 50−80 individual transients (laser "shots") averaged for each spectrum. Data were analyzed with the Ciphergen ProteinChip Software using the All-in-One Peptide Standard (Ciphergen) or internal peptide standards synthesized at our facility.

Measurement of Carbonyls in CSE. Detection and quantification of aldehydes and ketones present in CSE were determined by HPLC following derivatization with 2,4-dinitrophenyl-hydrazine and extraction with pentane as previously described by several laboratories (*33*−*36*). Standard curves were generated by reacting a mixture of commercially obtained aldehydes (Sigma) at known concentrations under identical conditions.

Results

Characterization of DHDC Adducts Derived from MDA and Monofuctional Aldehydes. Before evaluating the role of aldehydes in cigarette smoke, peptides were reacted with MDA and pure monofunctional aldehyde to determine the capability of each reagent combination to produce the predicted fluorescent adduct and to produce authentic standards for later experiments. Following modification of the peptide, under each specific reaction condition, the peptide products were assessed for changes in mass (MALDI-TOF), for the ability to fluoresce at the appropriate wavelength, and for retention time in a reverse phase HPLC column. Products that displayed strong fluorescence emission at 465 nm, when excited with 400 nm light, and that coeluted with a strong 214 nm absorbing peak (peptide bond) were presumed to be DHDC-adducted peptides, which was verified by mass spectroscopy of HPLC pure material. As illustrated in Figure 1, MDA and a monofunctional aldehyde may react with an amine to form DHDC adducts with the monofunctional aldehyde responsible for the composition of the R_1 group. Table 1 summarizes the expected structure of the R_1 group when forming the fluorescent DHDC derivatives. Because one carbon from the monofunctional aldehyde is incorporated into the DHDC ring structure, the corresponding R_1 group is simply a truncation of the CHO group from the monofunctional aldehyde's structure. Note that the change in peptide mass (Δ mass) and net mass of each adduct−peptide combination have been calculated to provide a guide for the interpretation of MALDI-TOF data.

Molecules with masses corresponding to the predicted mass for each DHDC peptide combination were observed in the appropriate reaction mixtures, and the data for each of the reaction conditions are summarized in Figure 2. In the control reaction, peptide plus buffer, a strong signal was observed at 1060.2 Da, which matched the predicted peptide mass of 1060 Da, and a weaker signal at 1082.4 Da corresponds to the mass of a peptide−sodium complex (Δ of 22 Da), which is a common artifact for MALDI-TOF experiments in systems utilizing sodium-based buffers. In the reactions containing MDA and one of the monofunctional aldehydes, strong signals of 1180.3, 1194.2, 1207.7, 1222.0, and 1256.6 Da were observed, which corresponded very well to the predicted masses of peptides modified with 1,4-dihydropyridine-3,5-dicarbaldehyde (HDHDC), MDHDC, 4-ethyl-1,4-dihydropyridine-3,5-dicarbaldehyde (EDHDC), 4-propyl-1,4-dihydropyridine-3,5-dicarbaldehyde (PDHDC), and 4-benzyl-1,4-dihydropyridine-3,5-dicarbaldehyde (ZDHDC) adducts, respectively, as specified in Table 1. These data clearly indicate that the peptide modifications occurring in the MDA monofunctional aldehyde reactions have masses exactly correlating with those predicted by DHDC adducts.

While we focused our study on fluorescent hybrid adducts, the reaction of the peptide with only MDA resulted in the production of modified peptides. Only one mass peak corresponded to the predicted mass of the peptide modified with a MDA Schiff's base, with a 1138.7 Da signal, which is indicative of the presence of a peptide−MDA−sodium (1060 + 56 + 22 = 1138) Schiff's base. In addition, weaker mass signals in the aldehyde containing reactions were observed, which do not have any obvious correlation to any predicted peptide modification.

Peptides modified with the aldehyde mixtures were also separated by HPLC to assess the fluorescent properties of the individual products, to measure ultraviolet absorbance in the peptide bond absorbing wavelength (214 nm), and to provide for the isolation of pure peptide with specific adducted products from the reaction mixture. Figure 2 summarizes the HPLC data collected for the relevant peptide−aldehyde reactions. As expected, the control reaction composed of only peptide and buffer produced no fluorescent product and revealed only the single parent peptide peak at 214 nm with a retention time of 20.18 min. The incubation of the peptide with MDA, under the conditions specified in the Materials and Methods, unexpectedly produced a fluorescent peak with a retention time of 36.8 min; however, the fluorescent product did not coelute with any material absorbing at 214 nm, indicating that this fluorescent product was not composed of peptide (i.e., could not be an aldehyde−peptide adduct). In addition, the reaction of only MDA with the peptide appeared to produce two nonfluorescent protein modifications with retention times of 25.88 and 31.93 min, which were not further characterized due do limiting the scope of this project to the fluorescent products. When a monofunctional aldehyde was included in the peptide−MDA reaction, a strongly fluorescent product was produced, which coeluted with a 214 nm absorbing product, which indicates that the reaction resulted in derivatization of the peptide with a fluorescent adduct. The coelution of a fluorescent and 214 nm absorbing product was particularly striking in the experiments containing peptide and MDA with AA, PA, BA,

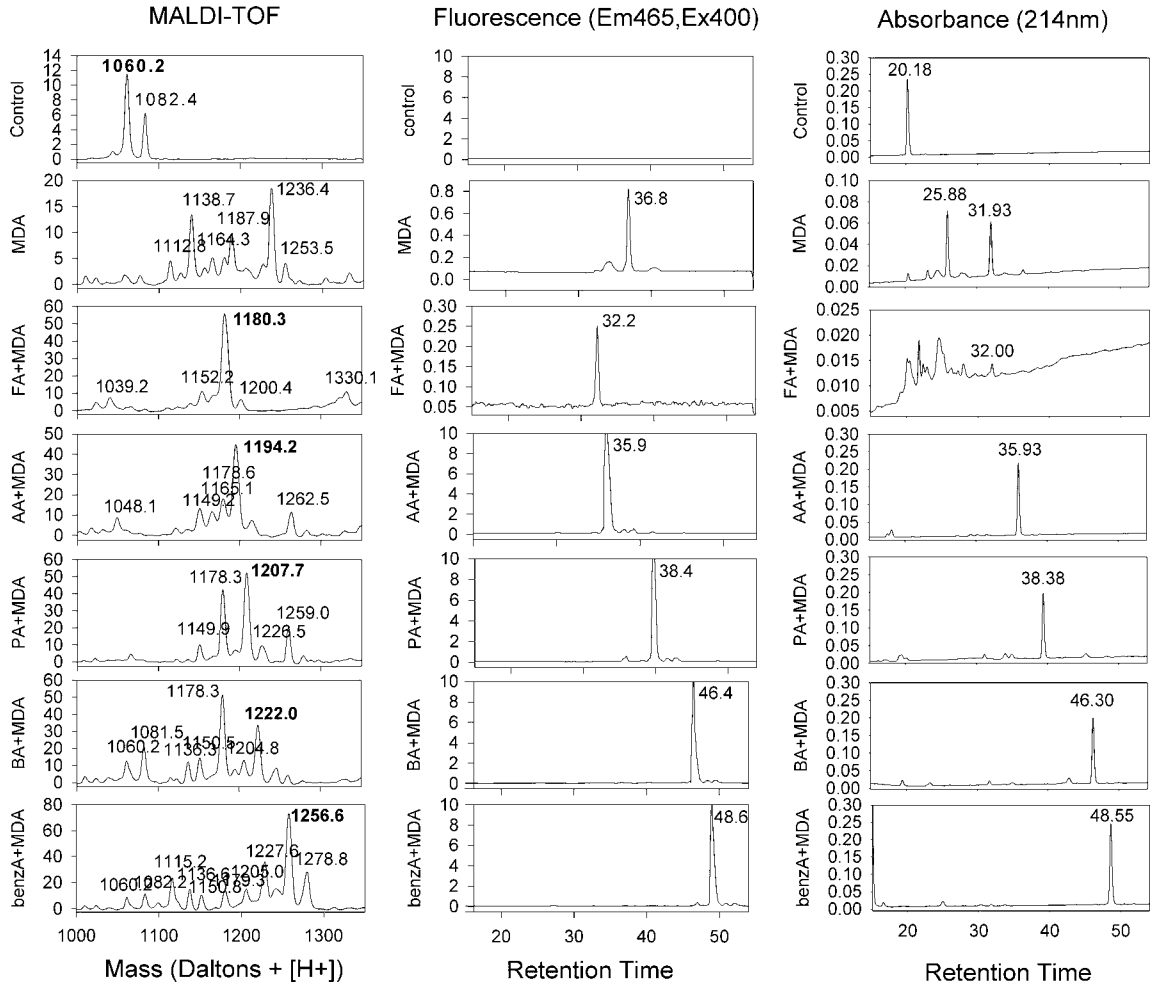

Figure 2. Modification of the test peptide with fluorescent DHDC adducts. The test peptide was exposed to control, MDA, FA and MDA, AA and MDA, PA and MDA, BA and MDA, or ZA and MDA as described in the Materials and Methods. The reactions were then evaluated by MALDI-TOF MS and HPLC with simultaneous ultraviolet (214 nm) and fluorescent (excitation 400 nm/emmission 465 nm) detection. MALDI-TOF data with peaks correlating to the predicted products indicated in Table 1 are in bold face, and the retention times of major products from the HPLC column are indicated.

or ZA, which essentially saturated the fluorescent detector and produced strong 214 nm absorbing peaks of 0.20−0.25 absorption units. While the reaction containing peptide, MDA, and FA produced a single fluorescent peak, the detection of multiple peaks at 214 nm indicated a plethora of products of which only one coeluted with the fluorescent peak. As a verification of the identity of the fluorescent peptide products, the fluorescent peaks were purified at an analytical scale by collection of HPLC fractions followed by lyophyllization of frozen fractions as described in the Materials and Methods. The mass of each product was measured by MALDI-TOF, and each corresponded to the respective DHDC−peptide adduct. HPLC pure adducted peptides were then dissolved in water, and the fluorescent properties were compared to the values obtained from identical modifications of methylamine. Because the maximum emission and excitation wavelengths correlate with identical modifications of methylamine (Table 2), the HPLC analysis detected coeluting peptide and fluorescent products, and the mass of each HPLC pure sample matched the predicted mass listed in Table 1; the identity of each fluorescent peak was confirmed as the predicted DHDC−peptide adducts.

Reaction of Peptide with CSE and MDA. The test peptide was exposed to chemicals derived from mainstream cigarette smoke to determine if any of the DHDC adducts were produced. CSE has been widely utilized for in vitro and in vivo studies related to cigarette smoke composition and the biological consequences of cigarette smoking, so the water soluble components of cigarette smoke were harvested by making an extract as described in the Materials and Methods. As seen in Figure 3, the exposure of peptide to CSE alone did not result in the formation of any fluorescent products; however, the addition of MDA to the CSE−peptide reaction produced one major and several minor fluorescent products. Two of the fluorescent products coeluted with strong 214 nm absorbing peaks and displayed retention times identical to the HDHDC- and MDHDC-adducted peptides. Unfortunately, we were not able to directly detect the mass of any modified peptides by MALDI-TOF in the CSE-containing samples. This was likely due to chemical components present in CSE interfering with crystallization of the peptide−CHCA matrix, which is required for effective MALDI-TOF mass spectroscopy. To overcome the interference of CSE, the reaction was replicated on

Table 2. Fluorescent Properties of DHDC-Adducted Methylamine and Peptide[a]

	HDHDC	MDHDC	EDHDC	PDHDC	ZDHDC
methylamine	398/468	398/465	398/465	398/466	392/452
peptide	397/469	395/463	396/466	398/468	393/450

[a] Excitation and emission peaks (excite/emit) are shown as described in the Materials and Methods.

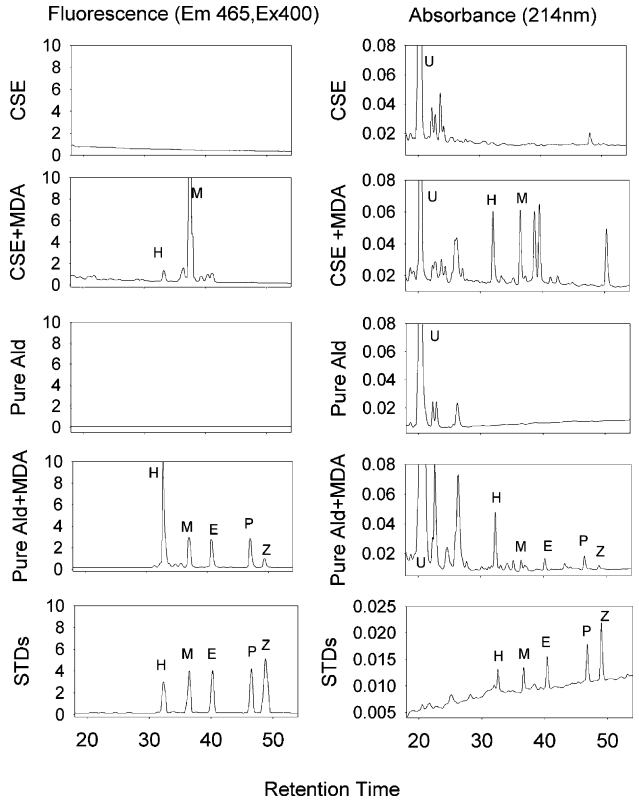

Figure 3. Modification of the test peptide with fluorescent adducts derived from CSE, and MDA was evaluated by HPLC. Samples were treated and separated by HPLC with simultaneous ultraviolet (214 nm) and fluorescent (excitation 400 nm/emmission 465 nm) detection as described in the Materials and Methods. The test peptide was exposed to CSE, CSE and MDA (CSE + MDA), a mixture of pure aldehydes (pure Ald), or a mixture of pure aldehydes and MDA (pure Ald + MDA). CSE was diluted 1:10 in PBS before use in the reactions. When indicated, MDA was included at 1 mM, and the mixture of pure aldehydes was composed of FA, AA, PA, BA, and benzA, each at 0.2 mM. A mixture of authentic standards (STDs) composed of HPLC-purified HDHDC-, MDHDC-, EDHDC-, PDHDC-, and ZDHDC-adducted peptides was run under identical conditions for identification of the fluorescent products.

a larger scale utilizing 10 mg of peptide, and the two major fluorescent products were purified by HPLC following concentration of the sample by freeze drying. MALDI-TOF analysis of the purified samples verified that the two fluorescent products, which coeluted with the HDHDC and MDHDC standards, did indeed have masses consistent with the HDHDC and MDHDC products. As additional controls, the reactions between the peptide and a mixture of monofunctional aldehydes or the peptide and an aldehyde mixture containing MDA were included. The mixture of monofunctional aldehydes did not produce any fluorescent products, but as seen with the CSE, the addition of MDA produced several fluorescent products, which coeluted with strong 214 nm absorbing peaks and displayed retention times identical to the HDHDC-, MDHDC-, EDHDC-, PDHDC-, and ZDHDC-adducted peptides. These data demonstrate that CSE is capable in the presence of MDA of producing stable DHDC adducts and that the HDHDC and MDHDC products are generated with the chemical components found in cigarette smoke.

Quantification of Carbonyl Groups in CSE. The aldehydes present in the CSE produced at our facility were assayed for correlation with the synthesis of specific DHDC adducts. As seen in Figure 4, CSE contains

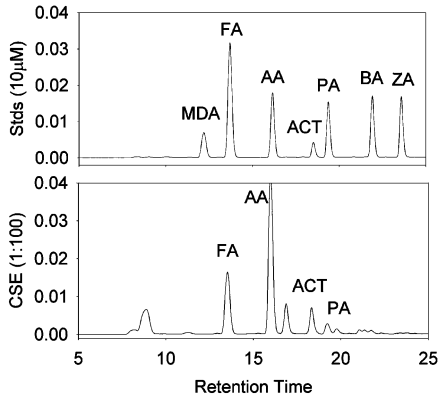

Figure 4. Quantification of cabonyl groups in CSE. Aldehyde levels in the CSE were assayed by HPLC following derivatization with 2,4-dinitrophenyl-hydrazine as described in the Materials and Methods. Standard curves for the assay were obtained by including reactions with mixtures of MDA, FA, AA, ACT, PA, BA, and ZA at known concentrations. A standard reaction containing 10 μM of each is included in the upper graph. Representative results for a 1:100 dilution of CSE are shown in the lower graph. The mean concentrations for FA, AA, and PA were 0.071 $\pm$ 0.009, 4.79 $\pm$ 0.58, and 0.013 $\pm$ 0.005 mM, respectively. BA and ZA were not detected. Data represent the means and standard deviations of three determinations.

detectable quantities of FA, AA, and PA at 0.071 $\pm$ 0.009, 4.79 $\pm$ 0.58, and 0.013 $\pm$ 0.005 mM, respectively, which are representative of the per cigarette values reported in the literature (*37*−*41*). BA and ZA were not detected.

Discussion

Results of this study clearly indicate that monofunctional aldehydes in the presence of MDA can react with internal lysine residues present in a peptide to produce HDHDC-, MDHDC-, EDHDC-, PDHDC-, and ZDHDC-adducted molecules. These adducts occur on the ϵ-amine of lysine and appear to be identical to the DHDC modifications of methylamine, which were described by Kikugawa (*23*). While extensive evidence has been presented in the literature for the formation of the MDHDC adduct on internal lysine residues of proteins (*21*, *42*−*46*), this represents the first description of HDHDC, EDHDC, PDHDC, or ZDHDC adducts forming on a protein or peptide model.

Of particular interest to our combined laboratories is the observation that aldehydes present in cigarette smoke, captured in the form of CSE, can react in combination with MDA to form HDHDC and MDHDC adducts on peptides. A simple analysis of the carbonyls, or aldehydes, in CSE indicate that AA is the most abundant monofunctional aldehyde present in CSE followed by FA. Because the HDHDC and MDHDC adducts are formed by a Hantzsch type reaction between MDA with FA or AA, respectively, a direct correlation between the monofunctional aldehydes present in CSE and the production of each DHDC adduct is easily explained. The absence of the EDHDC adduct, despite the detectable presence of propylaldehyde, is likely due to the small amount of propylaldehyde in our CSE preparation, the inability of propylaldehyde to compete effectively with the overwhelming excess of AA, or perhaps reaction kinetics that favor the smaller monofunctional aldehydes. Given these data, we hypothesize that the HDHDC and MDHDC modifications are likely to predominate in vivo following exposure to cigarette smoke.

The source of MDA, which is required for the synthesis of all DHDC adducts, is the decomposition of lipid peroxides, and the peroxidation of lipids is increased following exposure to cigarette smoke (*47*−*50*). Evidence for increased levels of MDA during smoke exposure includes increased plasma levels of thiobarbituric acid reactive substances (*51*, *52*), increased tissue levels of MDA (*53*−*55*), and the high level of pro-oxidants in cigarette smoke (*6*, *25*, *37*, *38*, *41*). The mechanisms for increased oxidative damage in lung have been proposed to include increased exposure to oxidants and pro-oxidants (*56*), recruitment of macrophage and neutrophils (*57*), dietary antioxidant (*58*) and glutathione (*59*) depletion, and radical production by inhaled small particles (*60*). In addition, recent unpublished data collected by our laboratories indicate that MDA, FA, and AA are present in exhaled breath condensates collected from individuals that smoke cigarettes, indicating that all of the precursors required for the synthesis of HDHDC- and MDHDC-adducted proteins are present in the lungs of smokers. However, additional studies will be required to confirm the presence of these adducts in vivo and to evaluate the importance of these adducts in disease.

Despite the lack of direct mechanistic evidence between DHDC adducts and disease, several reports in the literature suggest a putatively important role for similar chemical modifications in the development of several diseases. For example, the oxidation of LDL has been implicated in the development of cardiovascular disease (*61*−*63*), and exposure to cigarette smoke is thought to catalyze modifications of LDL, which facilitate increased uptake of LDL by macrophages (*64*−*68*). Most importantly, the oxidation of LDL has been shown to result in the derivatization of some lysine groups with the MDHDC adduct (*21*), and we suspect that exposure to cigarette smoke would result in a measurable increase in the abundance of the MDHDC, and likely HDHDC, modifications found on LDL due to an elevated exposure to precursor molecules such as FA, AA, and MDA. In addition, the modification of proteins with the MDHDC (or MAA) adduct has been suggested to promote alterations in normal immunological responses such as secretion of proinflammatory cytokines following uptake by macrophage or endothelial cells (*22*, *69*, *70*) and the alteration of immunological responses to adducted proteins such as the breaking of immunological tolerance (*46*, *70*−*75*). Should the disease modulating effects of DHDC adducts extend to the development of diseases associated with exposure to cigarette smoke, the future study of the role of DHDC adducts could provide profound insight into the mechanisms of which specific protein modifications lead to advanced disease states.

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