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Immunochemical Evidence Supporting 2-Pentylpyrrole Formation on Proteins Exposed to 4-Hydroxy-2-nonenal

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Previous model studies suggested the formation of lysine-based 2-pentylpyrroles as novel late adduction products formed upon exposure of proteins to the lipid peroxidation product 4-hydroxy-2-nonenal (HNE). Two 2-pentylpyrrole immunogens were prepared, one by treating keyhole limpet hemocyanin (KLH) directly with 4-oxononanal and the other by preformation of 6-(2-pentylpyrrol-1-yl)hexanoic acid from 6-aminocaproic acid and 4-oxononanal, followed by carbodiimide coupling to KLH. Pyrrole content and lysine modification in KLH were assayed independently. Following immunization of rabbits, antibody titer increased and plateaued over a 4 month period. The structural specificity of the IgG fractions of the antisera was evaluated through comprehensive competitive ELISA studies. These antibodies were used to verify the time-dependent appearance of the 2-pentylpyrrole epitope in protein exposed to HNE. Potential advantages of antibodies recognizing "advanced lipid peroxidation end products" over those recognizing "early" HNE adduction products are discussed.

Introduction

The increased awareness of the role of oxidative stress in degenerative disease has led to substantial current research on the nature of protein modification by reactive products of lipid peroxidation (*1*, *2*). Based on the identification of 4-hydroxy-2-nonenal (HNE)[1] as the "most cytotoxic aldehyde" released during peroxidation of linoleate and arachidonate fatty esters (*2*), several groups have focused on the suspected role of HNE in altered protein function which contributes to neurodegeneration (*3*−*5*) and in the oxidative modification of LDL underlying initial stages of atherosclerosis (*6*−*11*).

Although it was initially presumed that the major toxic HNE modification was associated with the formation of hemiacetal-stabilized Michael adducts of protein-based thiols (*2*), other studies led to the realization that HNE could form Michael adducts also with His imidazole and Lys ϵ-amino groups (*12*−*15*). We recently obtained structural verification that the first-formed amine-based HNE adducts which form rapidly represent simple amine Michael adducts and Schiff base Michael adduct 1:2 "cross-links" (*16*). Both polyclonal and monoclonal antibodies directed at the HNE Michael adducts have been prepared (*17*−*20*). In fact, the Michael adduct antibodies developed so far are not specific to the amino acid modified, but appear to recognize the pentyltetrahydrofuran structure of the Michael adduct hemiacetals. Recently, an antibody to the Schiff base Michael adduct "cross-link" has been reported (*21*).

In the case of low-density lipoprotein (LDL), modification by HNE results in a large increase in negative charge density (*6*−*8*), indicating that the major alteration involves charge neutralization of the many Lys ϵ-amino groups present in the protein component of the LDL particle. Our model studies revealed that, in contrast to the HNE Michael adduct of Cys thiol and His imidazole, the Lys ϵ-amino-derived HNE adducts were formed reversibly (*16*). In fact, the Schiff base Michael 1:2 adduct we isolated, in the presence of excess amine, underwent complete reversal to HNE when dissolved in aqueous buffer. Our ability to provide unambiguous structural information on the HNE adducts formed in the presence of amine came in part through characterization of the $NaBH_4$-reduced adducts, namely, the 3-amino-1,4-nonanediol in the case of the Michael adduct and 1,3-diamino-4-nonanol in the case of the 1:2 Schiff base Michael adduct (*16*). The fact that antibodies directed at HNE-modified LDL displayed very low antigenicity, unless the HNE-LDL was reductively stabilized ($NaBH_4$) prior to raising antibodies (*10*), is consistent with the reversible nature of HNE amine modification and suggests that the anti-HNE-LDL antibodies are recognizing principally 4-nonanol and 1,4-nonanediol modifications of lysine. It is unclear whether these modifications would form under physiological conditions, and thus to what extent the anti-borohydride-HNE-LDL antibodies are useful.

Our previous studies on the Paal−Knorr condensation of amines with 1,4-dicarbonyl compounds led us to recognize that initial Schiff base reactivity of HNE with amines could lead to a common intermediate in the pathway for pyrrole formation. In fact, we verified the formation of 2-pentylpyrroles upon long-term exposure of amines to HNE under physiomimetic conditions (*22*). Structural proof was obtained by independent synthesis via the Paal−Knorr condensation with 4-oxononanal. The

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[1] Abbreviations: DCC, 1,3-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; HOSu, *N*-hydroxysuccinimide; HOSu(SO_3)Na, *N*-hydroxysulfosuccinimide sodium salt; HNE, 4-hydroxy-2-nonenal; ON, 4-oxononanal; OP, 4-oxopentanal; DMF, *N*,*N*-dimethylformamide; PPC, 6-(2-pentylpyrrol-1-yl)caproyl; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; HSA, human serum albumin; COA, chicken ovalbumin; LDL, low-density lipoprotein; LGE_2, levuglandin E_2; ELISA, enzyme-linked immunosorbent assay; HRMS, high-resolution mass spectrometry; APT, attached proton test; TNBS, trinitrobenzenesulfonic acid; PBS, phosphate-buffered saline; ALPE, advanced lipid peroxidation end product.

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yield of pyrrole from HNE was low, however, and its formation in HNE-treated protein was too low to permit its detection by direct chemical means. Nonetheless, unlike the "early" Michael and 1:2 Schiff base Michael adducts formed by HNE, the pyrrole would represent a stable end product of HNE protein modification and would be a more valid contributor to the eventual irreversible HNE modification of LDL that results in Lys ϵ-amino group neutralization. This report focuses on the generation of polyclonal antibodies to the HNE-derived 2-pentylpyrrole and their use in verifying pyrrole formation in HNE-modified protein.

Experimental Section

General Methods. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Gemini 300 instrument. In all cases, tetramethylsilane or the solvent peak served as an internal standard for reporting chemical shifts. In the ^{13}C NMR line listings, attached proton test (APT) designations are given as (+) or (−) following the chemical shift. High-resolution mass spectra (HRMS) were obtained at 20 eV on a Kratos MS-25A instrument. Analytical and preparative thin-layer chromatography was performed using Merck silica gel 60 plates with 254 nm indicator. HNE, the HNE Michael adduct hemiacetals of N^{α}-acetyl-L-His and N^{α}-acetyl-L-Cys, and the reduced HNE Michael adducts of histamine and N^{α}-acetyl-L-Lys-NHCH$_3$ (N^{α}-acetyl-N^{τ}-3-(1,4-dihydroxynonyl)histamine and N^{α}-acetyl-N^{τ}-[1-(2-hydroxyethyl)-2-hydroxyheptyl]-L-lysine *N*-methylamide) were from a previous study (*16*), as was the LGE$_2$-derived protein-bound pyrrole, LGE$_2$-HSA (*23*). Bovine serum albumin (BSA), human serum albumin (HSA), and chicken ovalbumin (COA) were obtained from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, CA). HOSu(SO$_3$)Na was obtained from Pierce (Rockford, IL). All other organic chemicals were from Aldrich Chemical Co. (Milwaukee, WI), and reagents and solvents were AR grade. The dialysis tubing had a molecular weight cutoff of 14 000. For all ELISAs, unless otherwise noted, duplicates of each sample were run on the same plate.

6-(2-Pentylpyrrol-1-yl)caproic Acid (PPC-OH). 4-Oxononanal (1.73 g, 11 mmol) (*24*) and 2.6 g (20 mmol) of 6-aminocaproic acid were dissolved in 20 mL of CH$_3$CN/H$_2$O (1:1 v/v) solution. The reaction mixture was stirred for 48 h under nitrogen at room temperature. Then the reaction solution was concentrated and extracted with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ extracts were washed with H$_2$O, dried with Na$_2$SO$_4$, and evaporated to yield a yellowish-brown oil. The crude compound was purified by silica gel chromatography (EtOAc/hexane, 1:2 v/v, as eluent). Yield: 2.4 g (87%). ^{1}H NMR (CDCl$_3$) δ 0.92 (t, J = 6.8 Hz, 3H), 1.31−1.44 (6H), 1.66−1.79 (6H), 2.39 (t, J = 7.4 Hz, 2H), 2.53 (t, J = 7.8 Hz, 2H), 3.80 (t, J = 7.4 Hz, 2H), 5.87 (m, 1H), 6.06 (m, 1H), 6.57 (m, 1H). ^{13}C NMR (CDCl$_3$) δ 14.12(−), 22.62(+), 24.37(+), 26.26(+), 26.35(+), 28.65(+), 31.18(+), 31.84(+), 33.95(+), 46.20(+), 105.17(−), 106.64(−), 119.62(−), 133.21(+), 180.23(+). HRMS calcd for C$_{15}$H$_{25}$NO$_2$ 251.1887, found 251.1884.

6-(2-Pentylpyrrol-1-yl)caproic Acid *N*-Hydroxysuccinimide Ester. 6-(2-Pentylpyrrol-1-yl)caproic acid (250 mg, 1 mmol) and 121 mg (1.05 mmol) of HOSu were dissolved in 15 mL of CH$_2$Cl$_2$, and then 217 mg of DCC (1.05 mmol) was added. The reaction mixture was stirred for 4 h. The precipitate was filtered and washed twice with CH$_2$Cl$_2$. The combined filtrate and washings were evaporated to yield a brown residue which was purified by flash chromatography (EtOAc/hexane, 2:3 v/v, as eluent). Yield: 320 mg (92%). ^{1}H NMR (CDCl$_3$) δ 0.90 (t, J = 6.8 Hz, 3H), 1.31−1.41 (6H), 1.63−1.79 (6H), 2.50 (t, J = 7.4 Hz, 2H), 2.59 (t, J = 7.8 Hz, 2H), 2.83 (s, 4H), 3.78 (t, J = 7.4 Hz, 2H), 5.87 (m, 1H), 6.0 (m, 1H), 6.56 (m, 1H).

***N*-(2-Hydroxyethyl)-6-(2-pentylpyrrol-1-yl)caproamide.** A solution of the preceding active ester (35 mg, 0.1 mmol) and 12 mg of 2-hydroxyethylamine (0.2 mmol) in 10 mL of acetonitrile was stirred under nitrogen overnight, then evaporated to yield a brown residue. The residue was purified by preparative TLC (MeOH/EtOAc, 1:1 v/v, as mobile phase). Yield: 28 mg (95%). ^{1}H NMR (CDCl$_3$) δ 0.91 (t, J = 6.8 Hz, 3H), 1.17−1.39 (6H), 1.51−1.77 (6H), 2.19 (t, J = 7.5 Hz, 2H), 2.49 (t, J = 7.9 Hz, 2H), 3.33 (dt, J = 4.6 and 5.4 Hz, 2H), 3.70 (t, J = 5.3 Hz, 2H), 3.78 (t, J = 7.2 Hz, 2H), 4.40 (b, 1H), 5.86 (m, 1H), 6.05 (m, 1H), 6.47 (t, J = 4.6 Hz, 1H), 6.55 (m, 1H). ^{13}C NMR (CDCl$_3$) δ 14.09(−), 22.58(+), 25.36(+), 26.22(+), 26.62(+), 28.61(+), 31.16(+), 31.78(+), 36.33(+), 42.27(+), 46.20(+), 61.65(+), 105.08(−), 106.52(−), 119.62(−), 133.22(+), 174.11(+). HRMS calcd for C$_{17}$H$_{30}$N$_2$O$_2$ 294.2307, found 294.2314.

***N,N*-Bis(2-hydroxyethyl)-6-(2-pentylpyrrol-1-yl)caproamide.** 6-(2-Pentylpyrrol-1-yl)caproic acid (170 mg, 0.68 mmol), 71.5 mg of bis(2-hydroxyethyl)amine (0.68 mmol), 210.5 mg of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.02 mmol), and 117.4 mg of HOSu (1.02 mmol) were dissolved in 5 mL of CH$_2$Cl$_2$, and the reaction was monitored by TLC (MeOH/EtOAc, 1:1 v/v, as mobile phase). After 20 h, the precipitated *N*,*N*′-dicyclohexylurea (DCU) was filtered and washed twice with CH$_2$Cl$_2$. The combined filtrate and washings were evaporated to yield a yellow residue which was purified by preparative TLC (MeOH/EtOAc, 5:1 v/v, as mobile phase). Yield: 99 mg (43%). ^{1}H NMR (CDCl$_3$) δ 0.90 (t, J = 7.1 Hz, 3H), 1.31−1.39 (m, 6H), 1.63−1.73 (m, 6H), 2.39 (t, J = 7.4 Hz, 2H), 2.50 (t, J = 7.8 Hz, 2H), 3.26 (s, 2H), 3.54 (t, J = 5.0 Hz, 2H), 3.70 (t, J = 4.8 Hz, 2H), 3.77 (t, J = 6.3 Hz, 2H), 3.78 (t, J = 7.4 Hz, 2H), 3.84 (t, J = 5.0 Hz, 2H), 5.86 (m, 1H), 6.05 (m, 1H), 6.56 (m, 1H). ^{13}C NMR (CDCl$_3$) δ 14.09(−), 22.59(+), 24.90(+), 26.23(+), 26.62(+), 28.62(+), 31.29(+), 31.81(+), 33.36(+), 46.29(+), 50.39(+), 52.13(+), 60.84(+), 61.71(+), 105.01(−), 106.48(−), 119.73(−), 133.32(+), 175.19(+). HRMS calcd for C$_{19}$H$_{32}$N$_2$O$_3$ 338.2571, found 338.2572.

6-(2-Pentylpyrrol-1-yl)caproic Acid *N*-Hydroxysulfosuccinimide Ester. A solution of HOSu(SO$_3$)Na (217 mg, 1 mmol), 6-(2-pentylpyrrol-1-yl)caproic acid (PPC) (251 mg, 1 mmol), and DCC (227 mg, 1.1 mmol) in 10 mL of dry DMF was stirred at room temperature for 40 h. The precipitated DCU was removed by filtration and was washed with a small amount of dry *N*,*N*-dimethylformamide (DMF). The solvent was evaporated under high vaccum to give a brown residue which was washed with Et$_2$O (3 × 10 mL) to give a residue (yield 337 mg, 75%) which was used without further purification. The IR spectrum of the product (bands at 2932, 1786, 1740, 1636, 1232, and 1046 cm^{-1}) exhibited relatively strong and broad sulfonate bands at 1232 and 1046 cm^{-1}, which are predominant characteristics of the spectrum of the parent HOSu(SO$_3$)Na.

Preparation of ON-KLH Antigen. A standard solution of KLH was prepared at 4 °C by dialyzing the commercial suspension against 50 mM sodium phosphate buffer (pH 7.4) for 24 h, centrifuging down the insoluble material at 47800*g* for 15 min, and dialyzing the supernatant against 50 mM sodium phosphate buffer (pH 7.4) for 24 h. The protein concentration was determined by the Bio-Rad protein assay (Hercules, CA) as prescribed, using BSA as standard, and found to be 7.96 mg/mL. A solution of 7.5 mM 4-oxononanal (ON) and 31.8 mg of KLH in 6 mL of 50 mM sodium phosphate buffer (pH 7.2) and 2 mL of EtOH was incubated at room temperature for 4 h, followed by two successive 24 h dialyses against 1 L of 50 mM sodium phosphate buffer, pH 7.2. The extent of modification of lysines was determined by the trinitrobenzenesulfonic acid (TNBS) assay (*25*), and the concentration of protein pyrrole adduct was determined using Ehrlich's reagent (4-(dimethylamino)benzaldehyde) as described previously (*26*).

Preparation of PPC-KLH Antigen. The PPC HOSu(SO$_3$)Na active ester (20 mg, 0.044 mmol) and 2.5 mL of the standard KLH solution (19.9 mg) were mixed with 2.5 mL of water, and this solution was incubated at room temperature for 4 h. The solution was then dialyzed against 1 L of a 2:1 mixture (v/v) of 100 mM NH$_4$Cl and MeOH for 24 h and then again against 1 L of 50 mM sodium phosphate buffer (pH 7.4) for 24 h. The modified protein was characterized by TNBS and Ehrlich's assays.

Preparation of ON-BSA and ON-HSA. A solution of ON (7.5 mM) and either BSA or HSA (0.01 mM), final concentrations, in a mixture of 6 mL of 50 mM sodium phosphate buffer (pH 7.2) and 2 mL of EtOH was incubated at room temperature

for 4 h, and then dialyzed twice (24 h each) against 1 L of 50 mM sodium phosphate buffer (pH 7.2).

Preparation of PPC-BSA and PPC-HSA. A solution of the PPC $HOSu(SO_3)Na$ active ester (5.5 mM) and either BSA or HSA (0.01 mM), final concentrations, in 5 mL of sodium phosphate buffer (pH 7.4) was incubated at room temperature for 4 h, and then dialyzed against 1 L of a 2:1 mixture (v/v) of 100 mM NH_4Cl and MeOH for 24 h, and then again against 1 L of 50 mM sodium phosphate buffer (pH 7.4) for 24 h.

Preparation of OP-BSA. A solution of 4-oxopentanal (OP) (7.5 mM) and BSA (0.01 mM), final concentrations, in a mixture of 6 mL of 50 mM sodium phosphate buffer (pH 7.2) and 2 mL of EtOH was incubated at room temperature for 4 h, and then dialyzed twice (24 h each) against 1 L of 50 mM sodium phosphate buffer (pH 7.2). TNBS and Ehrlich's assays indicated a 48.8% extent of lysine modification and a pyrrole concentration of 0.28 μmol/mg of protein.

Immunization. The two immunogens used were ON-KLH (0.16 μmol of pyrrole groups/mg of KLH, 31.36 mg of KLH in 8 mL of pH 7.4 PBS) and PPC-KLH (0.25 μmol of pyrrole groups/mg of KLH, 19.6 mg of KLH in 5 mL of pH 7.4 PBS). Both immunogens were emulsified in Freund's complete adjuvant (400 μL). Two pairs of Pasturella free, New Zealand White rabbits (Hazelton) were inoculated intradermally into several sites on the back (125 μL) and rear legs (125 μL) for both immunogens. Booster injections of ON-KLH and PPC-KLH in Freund's incomplete adjuvant were given every 21 days. Antibody titers were monitored 10 days after each inoculation by ELISA as described below.

ELISA Analysis of Antibody Titers. For determination of ON-KLH and PPC-KLH antibody levels in rabbit blood serum, the corresponding BSA conjugates (ON-BSA and PPC-BSA, containing 26:1 and 49:1 pyrrole:protein molar ratios, respectively) were used as coating agents. The ON-BSA (100 μL solution containing 2.2 μg/mL in pH 7.4 phosphate-buffered saline (PBS)) and PPC-BSA (100 μL solution containing 0.7 μg/mL in pH 7.4 PBS) were added to each well of two sterilized Baxter ELISA plates. The plates were then incubated at 37 °C for 1 h in a moist chamber. After discarding the coating solution, each well was washed with PBS (3 × 300 μL), then filled with 1.0% chicken ovalbumin (COA) in PBS (300 μL), and incubated at 37 °C for 1 h to block remaining active sites on the plastic phase. Each well was washed with 0.1% COA in PBS (300 μL), and then 100 μL of rabbit serum from each bleeding diluted 1:10 000 with 0.2% COA in PBS or 0.2% COA in PBS without serum for a blank was dispensed into the sample wells. Normal rabbit (not injected with antigen) serum diluted as above was employed as a negative response control. The plate was covered and incubated at room temperature for 1 h with shaking. After discarding the supernatants and washing with 0.1% COA (3 × 300 μL), 100 μL of goat anti-rabbit IgG−alkaline phosphatase diluted 1:1000 with 1.0% COA was added to each well, and the plate was again incubated with shaking at room temperature for 1 h. After discarding the supernatant, the wells were washed with 0.1% COA (3 × 300 μL). Enzyme-linked antibody bound to the well was revealed by dispensing into each well 100 μL of disodium *p*-nitrophenyl phosphate (10 mg) in a solution (11 mL) containing glycine (50 mM), $MgCl_2$ (1 mM), and sufficient 6 M NaOH to raise the pH to 9.6. The plate was allowed to develop at room temperature until the maximum absorbance was judged appropriate, usually less than 30 min. Sample absorbances were then measured using a dual wavelength Bio-Rad 450 Microplate reader with detection at 405 nm relative to 655 nm.

IgG Fractionation. The crude ON-KLH antibody serum from the 53 days bleeding of rabbit 1 contained 40.1 mg/mL protein, and crude PPC-KLH antibody serum from the 74 days bleeding of rabbit 2 contained 38.7 mg/mL protein, as determined by absorbance at 280 nm. Seroclear (0.85 mL) was added to each of these two crude antibody serums (1.25 mL), and the mixtures were vortexed for 60 s. The mixtures were then centrifuged for 10 min at 3000 rpm (1900*g*), and the upper delipidated layers (aqueous phase, 1 mL) were removed and added to an equal volume of binding buffer (20 mM sodium phosphate, pH 7.0). The antibody solutions were filtered through 0.22 μm cellulose ester filters and loaded onto a protein G−Superose (Pharmacia) column. Non-protein-bound material was eluted with the binding buffer, and the bound IgG protein was eluted with 0.1 M glycine-HCl/0.15 M NaCl, pH 2.7. The eluates were collected into fractions (1 mL) containing pH 8.8 1.0 M Tris buffer (160 μL). The fractions containing IgG proteins were pooled and dialyzed against pH 7.4 PBS (0.02% NaN_3) for 24 h at 5 °C. The resulting solutions of anti-ON-KLH and anti-PPC-KLH (4.5 mL each) contained 0.74 and 0.99 mg/mL purified IgGs, respectively, as determined by absorbance at 280 nm (A_{280} = 1.35 for 1 mg/mL (*27*)). This corresponds to 6.6% and 11% of the IgGs in these two crude serums.

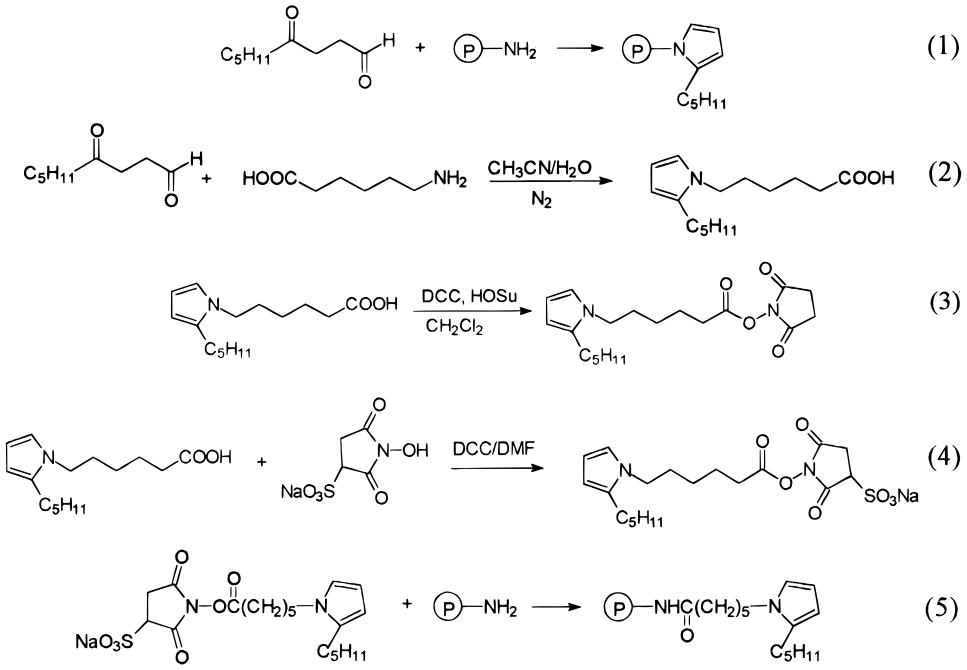
ELISA Characterization of Competitive Binding and Cross-Reactivity. For antibody binding inhibition studies to measure cross-reactivities, ON-BSA and PPC-BSA were used as coating agents for purified anti-ON-KLH and anti-PPC-KLH respectively. Each well of the plate was coated with the same BSA conjugate solutions (100 μL) used to measure antibody titers. For each inhibitor, a blank up to eight serial dilutions and a positive control containing no inhibitor were run. The plate was covered with a plastic lid and placed in an incubator at 37 °C for 1 h and then allowed to come to room temperature. After discarding the supernatant, each well was washed with pH 7.4 PBS (3 × 300 μL) and then blocked by incubating 1 h at 37 °C with 300 μL of 1% COA in pH 7.4 PBS. After coming to room temperature, the supernatant was discarded and the wells were rinsed with 0.1% COA in pH 7.4 PBS (300 μL). For each sample, the diluted sample solution (1:10, 150 μL) and aliquots (150 μL) of up to eight 1:5 serial dilutions with pH 7.4 PBS were incubated in test tubes at 37 °C for 1 h with desired ON-KLH or PPC-KLH antibody solutions (150 μL). The antibody solutions were prepared by adding the required amount of FPLC purified (protein G column) sera to 0.2% COA in pH 7.4 PBS (1:100 dilution for anti-ON-KLH and 1:1000 dilution for anti-PPC-KLH).

Blank wells were filled with 0.2% COA in pH 7.4 PBS (100 μL). Positive control wells were filled with the antibody solution (50 μL) and 0.2% COA solution (50 μL). To the rest of the sample wells, aliquots (100 μL, containing 50 μL of sample solution and 50 μL of desired antibody solution) of the serial dilutions of antibody−antigen complex were added. The plate was then incubated at room temperature on a shaker for 1 h. After discarding the supernatant, the wells were washed with 0.1% COA (3 × 300 μL), and then goat anti-rabbit IgG−alkaline phosphatase solution (100 μL) was added to each well. This enzyme-linked second antibody solution was prepared by diluting commercially available antibody solution (10 μL) with 1% COA (10 mL). The plate was then incubated at room temperature for 1 h while gently agitating on a shaker. After discarding the supernatant, the wells were washed with 0.1% COA (3 × 300 μL). To each well was then added a solution (100 μL) of disodium *p*-nitrophenyl phosphate (10 mg) in water (11 mL, pH adjusted to 9.6 using NaOH) containing glycine (50 mM) and $MgCl_2$ (1 mM). The plate was then incubated at room temperature for about 1 h until the absorbance levels reached an appropriate level. The absorbance in each well was measured with a dual wavelength Bio-Rad 450 Microplate reader with detection at 405 nm relative to 655 nm.

Absorbance values for duplicate assays were averaged and then scaled such that the maximum curve fit value is close to 100%. The averaged and scaled percent absorbance values were plotted against the log of concentration. Theoretical curves shown for each plot were fit to the absorbance data with a four parameter logistic function, $f(x) = (a - d)/[1 + (x/c)^b] + d$, using SigmaPlot 4.14 from Jandel Scientific Software (San Rafael, CA). Parameter a = the asymptotic maximum absorbance, b = slope at the inflection point, c = the inhibitor concentration at the 50% absorbance value (IC_{50}, reported in Table 2, vide infra), and d = the asymptotic minimum absorbance. If necessary, constraints were placed on the parameters, usually the values for "*a*" and/or "*d*". A Cartesian graph was then created that shows plots of the experimental data (points) and calculated curves.

Immunochemical Detection of Pyrrole in HNE-Treated HSA. To determine the dependence of pyrrole formation on

Scheme 1



HNE concentration (Figure 5), solutions of HSA (0.05 mM) and HNE (0−2 mM) in 50 mM pH 7.4 PBS were incubated for 5 h at 37 °C, followed by dialysis for 24 h at room temperature against 50 mM pH 7.4 PBS prior to ELISA studies using the ON-KLH antiserum as described above. To determine the time course of pyrrole formation (Figure 6), a solution of HSA (0.05 mM) and HNE (2 mM) in 10 mL of 0.2 M pH 7.4 PBS was incubated at 37 °C. At different time intervals 0.5 mL aliquots were removed and mixed with 0.25 mL of 2.5 M pH 7.4 PBS and 0.25 mL of 1 M $NaBH_4$ in methanol. Samples were kept at room temperature for 4−8 h and then refrigerated at 5 °C until conduct of the ELISA using the ON-KLH antiserum as described above.

Results

Strategies for Antibody Preparation. Two different approaches were used to obtain antibodies recognizing the HNE-derived pyrrole. The first involved direct treatment of protein with 4-oxononanal (ON) (eq 1, in Scheme 1). Model reactions with amines indicated that pyrrole formation occurred in high yield, but we could neither guarantee what fraction of Lys ϵ-amino groups modified by ON would correspond to the pyrrole nor exclude the possibility of other amino acids being modified. The second approach involved preformation of a 2-pentylpyrrole on a lysine-like tether by reaction of 6-aminocaproic acid with ON (eq 2, in Scheme 1), which permitted us to purify the pyrrole prior to carbodiimide-mediated conjugation to the protein. Both approaches had advantages and disadvantages. The direct ON treatment would result in the exact Lys ϵ-amino-derived pyrrole that would form from HNE, but we could not exclude the presence of non-pyrrole modifications. The 6-aminocaproyl-tethered approach guaranteed that every Lys ϵ-amino modification represented a 2-pentylpyrrole, but the latter moiety would now be separated from the protein backbone by the equivalent of two lysines end-to-end, a situation which could engender an immunogenic response distinct from that induced by the single lysine side chain-tethered pyrrole.

A second issue involved the methodology for protein conjugation of the pyrrolated 6-aminocaproic acid. Conjugation of N-derivatized 6-aminocaproic acid is a fairly common practice and often is accomplished by *in situ* coupling of the acid to the protein in aqueous buffer or mixed aqueous−organic solvent using a water soluble carbodiimide (e.g., EDC) in the presence of *N*-hydroxysuccinimide (HOSu) or *N*-hydroxysulfosuccinimide ($HOSu(SO_3^-)$) (*28*, *29*). We evaluated various reaction conditions for direct covalent attachment of 6-(2-pentylpyrrolyl)caproic acid (PPC) to BSA by assaying the final dialyzed protein by a combination of the TNBS assay (*25*) to determine loss of Lys ϵ-amino groups and Ehrlich's reagent (acidified 4-(dimethylamino)benzaldehyde) to determine protein-bound pyrrole (*26*). Direct coupling gave mixed results complicated by occasional precipitation and, more importantly, (i) modification of Lys ϵ-amino groups by the carbodiimide reagent (by comparing the TNBS reading when PPC was omitted from the reaction mixture) as well as (ii) substantial noncovalent but nondialyzable binding of PPC (by comparing the Ehrlich reading when EDC was omitted from the reaction mixture). The latter finding suggested to us the formation of tight salt bridges between PPC and the Lys ϵ-ammonium groups that survive dialysis.

For these reasons, we switched to utilization of a preformed HOSu or $HOSu(SO_3^-)$ active ester of PPC. In the former case, we were able to purify and completely characterize the active ester following DCC-mediated coupling (eq 3, Scheme 1), but reaction of the non-aqueous-soluble active ester with protein in buffer−DMF resulted in partial precipitation and in buffer−MeOH gave excessive Ehrlich readings (relative to the TNBS assay), indicating noncovalent (but nondialyzable) binding of either the active ester or the hydrolyzed PPC. Our most reliable results were obtained using the preformed $HOSu(SO_3^-)$ active ester (eqs 4 and 5, Scheme 1), even though the latter had to be utilized without purification (see Experimental Section). Even in this case, however, we observed some nondialyzable binding of PPC beyond that expected on the basis of lysines modified (per TNBS assay). We found that, by dialyzing the modified protein against aqueous methanolic NH_4Cl prior to dialysis against phosphate buffer, most of the excess Ehrlich's reading could be eliminated, presumably as a conse-

Table 1. Analytical Data on ON and PPC Modifications in Antigens, Coating Agents, and Competing Protein Ligands

	% Lys modified[a]	μmol/mg Lys modified[b]	μmol/mg pyrrole groups[c]
ON-KLH	43.6	0.22	0.16
PPC-KLH	48.0	0.24	0.25
ON-BSA	61.2	0.54	0.39
PPC-BSA	78.3	0.70	0.86
ON-HSA	62.2	0.55	0.36
PPC-HSA	74.1	0.66	0.97

[a] Of TNBS-detectable lysine: 100 − 100(control OD_{TNBS} − modified OD_{TNBS}), where OD_{TNBS} refers to background-corrected readings. [b] From column 1, assuming 500 nmol of Lys/mg of KLH, 59 mol of Lys/mol of BSA (FW = 66 430), and 59 mol of Lys/mol of HSA (FW = 66 437). [c] From the background-corrected Ehrlich reading based on A_{540} = 0.821 for 0.067 mM PPC.

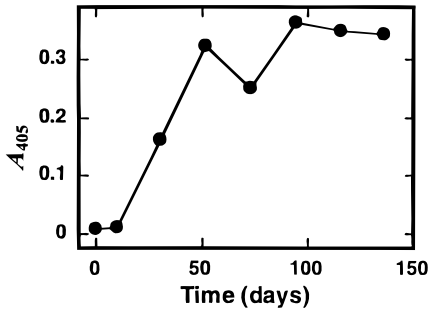


Figure 1. Antibody titer against 4-oxononanal−keyhole limpet hemocyanin (ON-KLH).

quence of competition of ion pairing of the ammonium ions with the noncovalently-bound PPC carboxylates.

The direct ON modification and PPC attachment was carried out (conditions described in Experimental Sections) with three proteins: (i) keyhole limpet hemocyanin (KLH) for obtaining immunogens, (ii) BSA for obtaining the corresponding ELISA coating agent, and (iii) HSA for obtaining a comparable competing ELISA antigen. Table 1 lists data on the extent (mole fraction available) of lysine modification of the six modified protein preparations as determined by TNBS assay and the extent (mole fraction) of pyrrole groups on the protein as revealed by Ehrlich's reagent. The latter value was obtained by comparison to the Ehrlich's absorbance observed for PPC itself. It can be seen that, for direct pyrrolation of protein using ON, the extent of Lys ϵ-amino groups exceeded that of pyrrole groups attached, whereas the opposite was true in the case of protein modification by the PPC active ester. This is consistent with some non-pyrrole modification using ON, as well as some non-lysine ϵ-amino-conjugated PPC or persistent noncovalently-bound (non-dialyzable) PPC in the case of the active ester method.

Inoculation of two New Zealand White rabbits each with the two KLH immunogens followed a schedule used previously (*23*), including booster injections given every 21 days. Appearance of antibody titer was monitored by ELISA, using the corresponding BSA-derived coating agent, chicken egg ovalbumin (COA) as the ELISA plate blocking agent, goat anti-rabbit IgG−alkaline phosphatase as secondary antibody, and *p*-nitrophenyl phosphate as chromogen. Preimmunization serum from each rabbit served as negative control for monitoring antibody appearance in each rabbit. Antibody titer increased over a period of 4−5 months and was found to maximize in about half this time period. No significant difference was observed between the two rabbits in each immunization protocol, and data are shown in Figures 1 and 2 for only one of the two rabbits for each antibody. Serum from

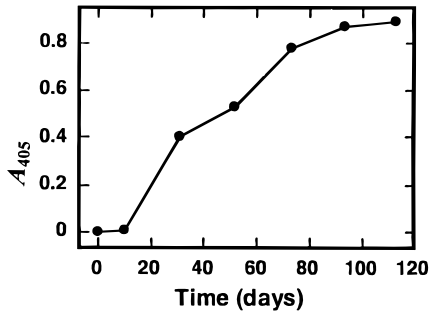


Figure 2. Antibody titer against 6-(2-pentylpyrrol-1-yl)-caproyl−keyhole limpet hemocyanin (PPC-KLH).

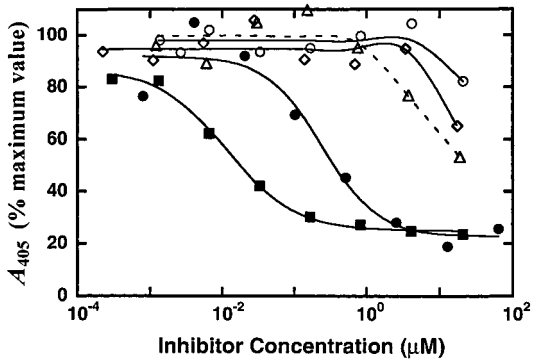


Figure 3. Inhibition curves showing cross-reactivity of the ON-KLH antibody for PPC-OH (■), PPC-HSA (●), OP-BSA (△), LGE_2-HSA (◇), and HNE-N^{α}-acetylcysteine (○) against ON-BSA as coating agent.

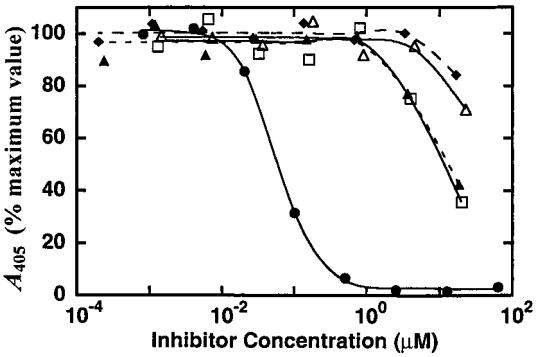


Figure 4. Inhibition curves showing cross-reactivity of the PPC-KLH antibody for PPC-HSA (●), PPC-$NHCH_2CH_2OH$ (□), OP-BSA (▲), and LGE_2-HSA (♦) against PPC-BSA as coating agent.

the 53 days bleeding of rabbit 1 (Figure 1) and the 74 days bleeding of rabbit 2 (Figure 2) was processed as described (Experimental Section), and the corresponding IgG fractions were obtained by protein G−Superose column purification.

Epitope Characterization of Anti-HNE-Pyrrole Antibodies. Epitope characterization of each of the two anti-KLH-pyrrole antibodies was conducted through competitive ELISA studies using the corresponding BSA-conjugated haptens as coating agents and the same secondary antibody detection system used for determination of antibody titer. Details are described in the Experimental Sections for construction of log plots which permitted computer fitting of theoretical sigmoidal binding curves (see Figures 3 and 4) and estimation of the IC_{50} for each of the competing antigens chosen (Table 2). For weak competitors which failed to display 50% inhibition at the maximum concentration tested (17−23 μM), Table 2 reports the % inhibition seen at this point. For the protein-bound haptens, the IC_{50} values refer to the

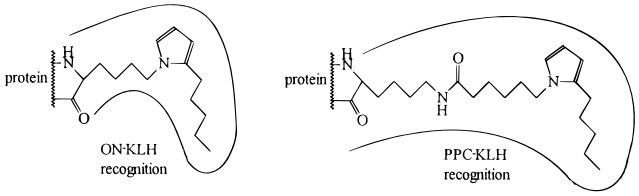
Table 2. HNE-Pyrrole Antibody Specificity

	ON-KLH Antibody (ON-BSA Coating)	PPC-KLH Antibody (PPC-BSA Coating)
	IC_{50} (μM) or Inhibition (percent at μM)	
PPC-BSA, protein = BSA	0.36	0.053
PPC-HSA, protein = HSA	0.24	0.045
ON-BSA, R = C_5H_{11}	0.79	0.90
ON-HSA, R = C_5H_{11}	1.12	3.50
OP-BSA, R = CH_3	19	13
PPC-Z, Z = $NHCH_2CH_2OH$	0.073	10
Z = $N(CH_2CH_2OH)_2$	0.044	20
Z = OH	0.012	16
LGE_2-HSA	35% at 17	18% at 17
HNE-N^{α}-acetylhistidine	25% at 20	30% at 20
HNE-N^{α}-acetylcysteine	18% at 20	19% at 20
HNE-N^{α}-acetylhistamine ($NaBH_4$-reduced)	0% at 22.5	29% at 22.5
HNE-N^{α}-acetyllysine N-methylamide ($NaBH_4$-reduced)	0% at 21	0% at 21

effective concentration of the pyrrole hapten as determined by the Ehrlich assay (see above).

The first two series of entries in Table 2 show that the PPC- and ON-derived BSA/HSA antigens were, as expected, more potent in inhibiting binding of the *corresponding* antibody rather than the *noncorresponding* antibody to their respective coating agents. At the same time, however, the PPC-BSA/HSA antigens were better inhibitors than the ON-BSA/HSA antigens toward both antibodies. These two findings together indicate that antibody recognition involves more than the common 2-pentylpyrrole moiety present in both antigens. We propose that each antibody optimally recognizes the full structural segment which extends from the protein backbone as shown in Scheme 2. The finding that the highest affinity displayed in this series was that of the PPC-KLH antibody for its own PPC-BSA/HSA antigen most likely reflects the more extensive surface contact area relative to the ON-KLH case, arising from the additional aminocaproyl spacer. Evidence that the PPC-KLH antibody recognizes both aminocaproyl segments between the pyrrole and the protein backbone is its low affinity for ON-BSA/HSA, which contains only one such segment. On the other hand, the more limited recognition domain of the ON-KLH antibody can "fit" either PPC- or ON-derived antigens, thus explaining the relative lack of discrimination of this antibody for the two antigen types. Interestingly, BSA pyrrolated with 4-oxopentanal (OP) was recognized 24-fold more weakly than 4-oxononanal−BSA (ON-BSA) by the ON-KLH antibody, indicating a pronounced specificity for the 2-pentyl as opposed to 2-methyl substituent on the pyrrole ring (even the nonmatching PPC-KLH antibody displayed a 14-fold affinity difference).

Scheme 2



The third series of entries in Table 2 conveys competitive binding data for non-protein-bound low molecular weight 2-pentylpyrroles. Based on the fact that the compounds shown represent 6-aminocaproic acid derivatives, one might naively have expected the PPC-KLH antibody to exhibit the greater affinity. However, the affinities were actually much greater for the ON-KLH antibody. The reason for this becomes clear upon reference to the data on the protein-bound haptens discussed above, which indicated that the PPC-KLH antibody recognizes a more extensive structure than does the ON-KLH antibody. Thus, 6-(2-pentylpyrrol-1-yl)caproic acid (PPC-OH) itself, even though this was the moiety attached to protein for raising the PPC-KLH antibody, best matches the recognition domain of the ON-KLH antibody. The hydrophilic amide derivatives of PPC-OH also displayed strong, albeit weaker, affinity for the ON-KLH antibody. For the PPC-KLH antibody, the relatively low affinity displayed by PPC-OH and its amide derivatives must reflect the fact that these derivatives are missing the "left-hand" recognition segment (lysine side chain) of the PPC-KLH antibody (Scheme 2). The surprisingly large relative affinity difference (10^3-fold) between the two antibodies observed for PPC-OH itself (an anion at physiological pH) must indicate that the PPC-KLH antibody can hardly tolerate the presence of the polar carboxylate in the midsection of its recognition domain.

In marked contrast to the 2-pentylpyrrole-based antigens (either protein-derived or low molecular weight versions), a structurally divergent pyrrole antigen (LGE_2-HSA), which arises from protein modification by a distinct product of lipid peroxidation (*23*), displayed only very weak binding to either of the antibodies raised in the present study. Also, the HNE-derived Michael adducts of both N^{α}-acetyl-L-His and N^{α}-acetyl-L-Cys, which are equally well recognized by the *only other epitopically defined* anti-HNE antibody currently in wide use (*19*), also display very weak affinity for the anti-pyrrole antibodies raised here. Competing ligands representing the borohydride-reduced forms of HNE-derived His and Lys Michael adducts, which are presumably the structures recognized by the borohydride-reduced anti-HNE-LDL antibodies raised previously (*10*), displayed little or no detectable affinity for our anti-pyrrole antibodies.

Immunochemical Confirmation of Pyrrole Formation in HNE-Treated Protein. Since the ON-KLH antibody, although exhibiting weaker affinities, appears to recognize more selectively the actual pyrrole-containing protein modification that would form from reaction with HNE, i.e., pyrrole formation directly at the Lys ϵ-amino group, we used this antibody to determine the level of pyrrole generated upon treatment of proteins with HNE. We exposed HSA as a prototypical protein to varying concentrations of HNE (up to 2 mM) for 5 h,

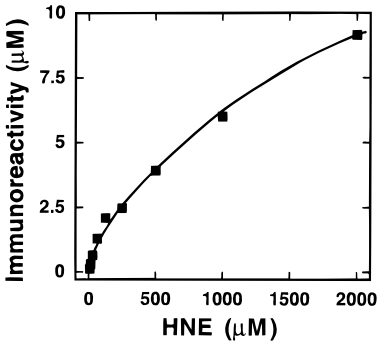


Figure 5. Pyrrole immunoreactivity generated in the reaction of HNE with HSA at 37 °C, pH 7.4, as a function of HNE concentration.

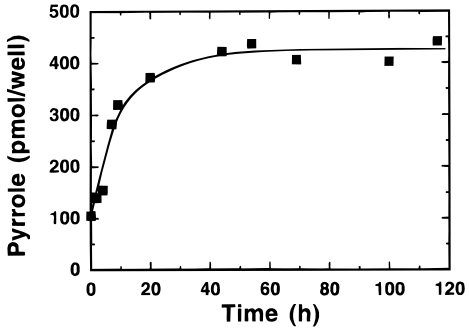


Figure 6. Pyrrole immunoreactivity generated in the reaction of HNE (2 mM) with HSA at 37 °C, pH 7.4, at various time intervals before $NaBH_4$ quenching.

followed by dialysis for 24 h to remove unbound HNE. Recognition by the ON-KLH antibody increased with increasing HNE concentration, as shown in Figure 5. The 5 h time point was chosen based on a previous report that immunoreactivity toward the anti-HNE-Michael adduct antibody plateaued by this time (*18*). However, since our model studies suggested that pyrrole formation might be a slow process *following initial attachment* of HNE to the lysine ϵ-amino group, the level of pyrrole immunoreactivity indicated in Figure 5 may represent a substantially longer reaction time than the 5 h predialysis incubation period.

In order to determine the time course of pyrrole formation, we exposed HSA to 2 mM HNE and quenched aliquots at various times by diluting with buffer and reacting with $NaBH_4$ for 1 h prior to dialysis. Our objective was to reduce unreacted HNE as well as any reducible HNE-protein intermediates (e.g., the Lys ϵ-amino Schiff base) that might otherwise evolve to pyrrole during dialysis. In this way, the level of pyrrole detected immunochemically would represent strictly the level of pyrrole present at the time of the borohydride quench. The quench conditions were chosen based on the minimal concentration of $NaBH_4$ needed to achieve essentially complete reduction of HNE in 1 h at pH 7. The results (Figure 6) indicate that pyrrole formation rises nearly linearly for 5 h, then begins to plateau, and reaches a maximal level by about 24 h. The significant level of pyrrole at the nominal 0 h reaction time represent pyrrole formation that occurs during the 1 h it takes $NaBH_4$ to reduce all the HNE. By comparing the maximal level of 2-pentylpyrrole immunoreactivity achieved in the HNE-HSA treatments (Figures 5 and 6) to that which forms in 4-oxononanal-treated HSA (of known quantity based on the Ehrlich assay), it is possible to estimate a yield of 0.8−1.0% pyrrole based on available Lys ϵ-amino groups in the protein. Direct Ehrlich assay of the modified HSA corresponded to 1.8−2% pyrrole based on total Lys content, regardless of whether the sample was dialyzed directly or first treated with $NaBH_4$. However, the absorbances from which this estimate was made were very low and thus not highly accurate. The TNBS assay, on the other hand, indicated modification of 32% of available Lys by the HNE treatment in the case of direct dialysis or 49% of available Lys in the case of $NaBH_4$ treatment prior to dialysis, indicating that the pyrrole corresponds to only a small fraction of the modifications induced by HNE.

Discussion

Based on the current interest in determining the nature of HNE-derived modifications which appear on LDL and other proteins under physiological conditions of oxidative stress, a significant effort has been made to develop analytical and immunochemical approaches which permit identification of the amino acid residues modified and the structure of the adducts. Although early work emphasized the importance of Cys sulfhydryl modification, the large shift in isoelectric point which accompanies HNE modification of LDL was interpreted to represent mainly modification of Lys residues. Stadtman and coworkers utilized a combination of (2,4-dinitrophenyl)-hydrazine reactivity and tritium incorporation from $[^3H]NaBH_4$ to conclude that the major reaction between HNE and protein-based amino groups, that occurs *at short reaction time*, involves Michael-type adducts (*12*−*15*). This same group and more recent followup studies (*21*) have provided evidence also for the formation of a 1:2 Schiff base Michael adduct representing a lysine−lysine cross-link. However, our previous model studies (*16*), aimed at obtaining an unambiguous structural characterization of these straightforward HNE adducts, revealed that in contrast to the stable Michael adducts formed with His imidazole and Cys sulfhydryl side chains, both the simple Michael adduct and 1:2 Schiff base Michael adduct formed between HNE and *amines* are reversible. We thus concluded that such adducts are unlikely to constitute the robust type of adduct formed between HNE and protein-based amino group, and they are not the charge-neutralizing adducts which appear to predominate in HNE-treated LDL, nor the fluorescent structures that appear upon extended treatment with HNE.

In our efforts to focus on HNE amine adducts which would form nonreversibly and would result in neutralization of protein-based Lys side chains, we identified a 2-pentylpyrrole, which could be independently generated from treatment of an amine with 4-oxononanal (*22*). Based on the multistep nature of the condensation which leads to pyrrole, we refer to this as an "advanced" type of HNE adduct to distinguish it from the "early" type of adduct represented by simple Schiff base formation or Michael addition. The expectation that advanced adducts would form more slowly over time, but would then represent stable end products, leads us to term the HNE-derived pyrrole as one example of an "advanced lipid peroxidation end product" (ALPE), in analogy to "advanced glycation end products" (AGE) that form in the reaction of amines with reducing sugars (*28*). It is worth pointing out that HNE is itself a vinylogous α-hydroxyaldehyde, and thus it is not unreasonable to expect that its chemistry might somewhat parallel that of reducing sugars.

In the present work, we prepared and epitopically characterized two polyclonal antibodies recognizing the

2-pentylpyrrole structure. The cross-reactivity ELISA inhibition studies indicate that the ON-KLH rather than PPC-KLH antiserum is more suited to recognize the Lys ϵ-amino-derived 2-pentylpyrrole that would be present in proteins exposed to HNE. The sensitivity of immunochemical detection permitted us for the first time to confirm and quantify the level of pyrrole generated in HNE-treated protein, a modification which we previously demonstrated only in model studies. The data allow us to estimate that, in HNE-treated HSA, only about one pyrrole per every two HSA protein molecules (each containing 59 amino groups) forms under the reaction conditions chosen. This low level would not be reliably estimated using Ehrlich's reagent, an indirect assay which might always be questioned due to the possible formation of non-pyrrole, Ehrlich's-positive adducts (of unknown structure).

Since the pyrrole represents an irreversible, albeit minor, product of HNE protein modification, we propose that the anti-HNE-pyrrole antibody may be a more relevant marker of permanent protein damage arising from adduction of products of lipid peroxidation than antibodies currently available which recognize HNE-derived Michael adducts, at least as far as lysine modification is concerned. In fact, the immunoreactivity generated in the reaction of HSA with HNE increased for a longer time than that found previously (*18*) using the anti-HNE-Michael adduct antibody, consistent with our classification of the pyrrole as one example of an ALPE. Certainly, the anti-HNE-pyrrole antibody will be more useful for studying lysine modification in HNE-treated or oxidatively modified-LDL than the previously available anti-HNE-LDL antibodies, which were raised against borohydride-reduced adducts, and which displayed poor recognition of unreduced samples. With the anti-HNE-pyrrole antibody in hand, we are in a good position to study variation in reaction conditions that might result in increased yield of pyrrole. More importantly, these antibodies provide powerful tools to investigate the presence of irreversible HNE modification in physiological studies relating oxidative stress and degenerative disease.

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