Specific Adducts Formed through a Radical Reaction between Peptides and Contact Allergenic Hydroperoxides

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The first step in the development of contact allergy (allergic contact dermatitis) includes the penetration of an allergy-causing chemical (hapten) into the skin, where it binds to macromolecules such as proteins. The protein−hapten adduct is then recognized by the immune system as foreign to the body. For hydroperoxides, no relevant hapten target proteins or protein−hapten adducts have so far been identified. In this work, bovine insulin and human angiotensin I were used as model peptides to investigate the haptenation mechanism of three hydroperoxide haptens: (5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (Lim-2-OOH), cumene hydroperoxide (CumOOH), and 1-(1-hydroperoxy-1-methylethyl) cyclohexene (CycHexOOH). These hydroperoxides are expected to react via a radical mechanism, for which 5,10,15,20-tetraphenyl-21*H,*23*H*-porphine iron(III) chloride (Fe(III)TPPCl) was used as a radical initiator. The reactions were carried out in 1:1 ethanol/10 mM ammonium acetate buffer pH 7.4, for 3 h at 37 °C, and the reaction products were either enzymatically digested or analyzed directly by MALDI/TOF-MS, HPLC/MS/MS, and 2D gel electrophoresis. Both hydroperoxide-specific and unspecific reaction products were detected, but only in the presence of the iron catalyst. In the absence of catalyst, the hydroperoxides remained unreacted. This suggests that the hydroperoxides can enter into the skin and remain inert until activated. Through the detection of a Lim-2-OOH adduct bound at the first histidine (of two) of angiotensin I, it was confirmed that hydroperoxides have the potential to form specific antigens in contact allergy.

Introduction

Contact sensitization and its clinical manifestation, allergic contact dermatitis (ACD[1]), are caused by low molecular weight compounds (haptens) which are able to penetrate into the skin. It is generally accepted that the haptens must bind to high molecular weight compounds in the skin to become immunogenic, and the formation of immunogens is normally considered to take place between an electrophilic hapten and nucleophilic moieties in amino acid side chains of skin proteins (*1*). In many cases, the prerequisite for this interaction is the chemical activation of the compounds to more reactive species either by autoxidation on exposure to air or by metabolic activation (*2*).

Fragrance compounds are the second most common cause of contact allergy after nickel (*3*). The monoterpenes limonene from citrus and linalool from lavender, or otherwise obtained by chemical synthesis, are the most commonly used fragrance compounds in perfumes found in every day products (*4*). They are themselves very weak allergens but are activated to strong sensitizers by autoxidation (*5*−*9*). Their primary oxidation products, the hydroperoxides, are the major sensitizers in the oxidation mixture formed on air exposure. Hydroperoxides have not been shown to react via nucleophilic−electrophilic interactions. Instead, hydroperoxides are believed to form covalent bonds though a radical mechanism (*1, 2*). It has therefore been discussed that hydroperoxides do not form specific antigens but cause a general hypersensitivity, through an unspecific oxidation reaction. In contrast, both clinical and experimental studies have demonstrated specific responses to the hydroperoxides investigated (*10*). The involvement of carbon, alkoxy, and peroxyl radicals has been demonstrated using radical trappers and cross-reactivity studies (*11, 12*). However, a radical reaction pathway of hydroperoxides for haptenation of proteins has so far not been confirmed in studies on hapten−protein/peptide interactions.

The aim of the present study was to investigate the role of radicals in the formation of hapten adducts. This was done by investigating the products formed in the reaction between the known sensitizers (5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (**1**, Lim-2-OOH), cumene hydroperoxide (**2**, CumOOH), and 1-(1-hydroperoxy-1-methylethyl) cyclohexene (**3,** CycHexOOH)(Figure 1) and peptides modeling skin proteins.

Experimental procedures

Caution: *The following chemicals are sensitizing, thus skin contact should be avoided: limonene-2-hydroperoxide, cumene hydroperoxide, cyclohexene hydroperoxide, and carvone.*

Chemicals and Biochemicals. Bz-N-His-OMe was purchased from Bachem (Bubendorf, Switzerland). (5*R*)-2-Methyl-5-(prop-1-en-2-yl)cyclohex-2-enone (R-carvone, 98%), cumene hydroperoxide (CumOOH) Luperox CU90, 5,10,15,20-tetraphenyl-21*H,*23*H*-porphine iron(III) chloride (Fe(III)TPPCl), human angiotensin I, bovine insulin, trypsin, chymotrypsin, proteinase K, Boc-Tyr-OMe, and the MALDI matrix α-cyano-4-hydroxycinnamic acid (HCCA)

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[1] Abbreviations: ACD, allergic contact dermatitis; Fe(III)TPPCl, 5,10,15,20-tetraphenyl-21*H,*23*H*-porphine iron(III) chloride.

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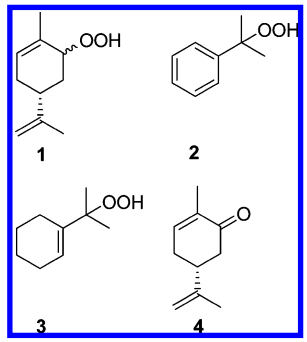

Figure 1. Chemical structures of compounds referred to in this article. (5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (**1**, Lim-2-OOH), cumene hydroperoxide (**2**, CumOOH), and 1-(1-hydroperoxy-1-methylethyl) cyclohexene (**3**, CycHexOOH), and carvone (**4**).

were obtained from Sigma-Aldrich (Stockholm, Sweden). The Sequazyme Peptide Mass Standards Kit Calibration mixture 2 from Applied Biosystems (Stockholm, Sweden) was used for the MALDI/TOF-MS calibration. The molecular weight markers used for native PAGE were PageRuler Unstained Protein Ladder #SM0661 and for SDS−PAGE PageRuler #SM 1811 (6.5−200 kDa), both from Fermenta (Helsingborg, Sweden). The C18 ZipTips and the Synergy 185 system used for purification of water were from Millipore (Billerica, USA). Organic solvents and other chemicals were from Sigma-Aldrich or BioRad (Sundbyberg, Sweden). Bovine insulin contains two amino acid chains with intra- and intermolecular disulfide bonds. They are marked here by superscripts: GIVEQC^{1}C^{2}ASVC^{1}SLYQLENYC^{3}N (α-chain) and FVNQHLC^{2}GSHLVEALYLVC^{3}GERGFFYTPKA (*β*-chain). The amino acid sequence of human angiotensin I is NRVYIHPFHL. The theoretical average *m*/*z* value of the quasi-molecular ion $[M + H]^+$ is 5733.49 amu for insulin and 1296.69 amu for angiotensin I.

Synthesis of Lim-2-OOH and CycHexOOH. The syntheses of Lim-2-OOH (*12*) and CycHexOOH (*11*) were performed as reported in the literature.

Stability of Lim-2-OOH. Lim-2-OOH (7.5 mg, 44.58 μmol) was dissolved in base-washed $CDCl_3$ (0.6 mL) and placed in an NMR tube. A stock solution of the Fe(III)TPPCl (3.1 mg, 4.40 μmol) in base-washed $CDCl_3$ (1.5 mL) was added (150 μL) to the NMR tube at $t = 0$. A control solution without catalyst was also prepared. The degradation of Lim-2-OOH was then followed over time by ^{1}H NMR at 400 MHz on a JEOL Eclipse+ 400 instrument. The endocyclic olefinic proton was used as a measure of the progress of the reaction.

MALDI/TOF-MS Analysis of the Reaction between Hydroperoxides and Angiotensin I or Insulin. Analyses were performed on a Voyager DE STR MALDI/TOF instrument (Applied Biosystems) using a 337 nm laser, with a 3 ns pulse width, and a 20 Hz repetition rate. Samples were mixed 1:1 (v/v) with matrix solution, and 1 μL of this mixture was applied onto the MALDI target. Instrument settings were optimized for the calibration mix at *m*/*z* ranges 100−2000, 700−9000, or 1400−9000, in positive reflector mode. Analyses were also performed on the corresponding control mixtures.

LC/MS Analysis of the Reaction between LimOOH and Boc-Tyr-OMe or Bz-N-His-OMe. The amino acid reactions and control mixtures were analyzed with LC/MS using an HPLC system (Shimadzu, Japan) consisting of two pumps (LC-10 ADvp), a degasser (DGU-14 A), a system controller (SCL-10 Avp), and an autoinjector (SIL-10ADvp) coupled to a triple quadrupole API 2000 mass spectrometer (Applied Biosystems/MDS Sciex, Canada). Typically, 20 μL of a 500 μL reaction or control mixture was injected onto a C18 100 × 2.1 mm YMC HPLC column and separated at a flow rate of 250 μL/min. The mobile phases were (A) 1 mM formic acid in water and (B) 1 mM formic acid in acetonitrile. A 20 min linear gradient from 10% B to 60% was employed, followed by an increase to 100% B in 1 min. The analytes were ionized by positive ESI with the following settings: curtain gas, 30 psi; collision gas, 6 psi; ion source gas 1, 30 psi; ion source gas 2, 40 psi; ion spray voltage, 4500 V; temperature, 200 °C; and collision energy, 30 or 40 V. Analyses were also performed on corresponding control mixtures.

Reactions of Hydroperoxides with Angiotensin I, Insulin, Boc-Tyr-OMe and Bz-N-His-OMe. The following stock solutions of angiotensin I, insulin, Boc-Tyr-OMe, BZ-N-His-OMe, Lim-2-OOH, CycHexOOH, CumOOH, and Fe(III)TPPCl were prepared: peptides (1 or 10 mg/mL) in 10 mM ammonium acetate (pH 7.4), amino acids (1 mg/mL) in EtOH, hydroperoxide (10 mg/mL) in EtOH, and Fe(III)TPPCl (0.034 mg/mL) in EtOH. New stock solutions of the hydroperoxides were made directly before starting each reaction while all other stock solutions were used either fresh or after storage at −20 °C. Reaction mixtures of peptide/hydroperoxide/Fe(III)TPPCl or amino acid/hydroperoxide/Fe(III)TPPCl were prepared from the stock solutions at molar ratios of 1:10:0.1 (if not otherwise stated in the text) with a total reaction volume of 0.10−1.0 mL. The final solvent ratio in the mixtures was 1:1 (v/v) EtOH/buffer. The reaction was initiated by adding Fe(III)TPPCl and then allowed to proceed at 37 °C for up to 180 min in darkness. In parallel to every reaction, three control mixtures were prepared, containing peptide/hapten, peptide/Fe(III)TPPCl, or peptide alone. Equivalent control mixtures were prepared for the amino acids. The reaction mixtures were analyzed directly after the reaction time specified in the text and figures or stored at −20 °C. Analysis of the peptide reaction was performed as described above using MALDI/TOF-MS, while the amino acid reactions were analyzed using LC/MS.

Native PAGE of Insulin Reaction. The volumes of the reaction- and control mixtures were reduced to half using vacuum centrifugation (Savant SpeedVac, Techtum Lab, Umeå), where after the mixtures were diluted 1:1 (v/v) with 10 mM ammonium acetate buffer (pH 7.4). Each well on a 16% native polyacrylamide gel was then loaded with10 μL of 1:2 (v/v) sample/sample buffer, resulting in a total peptide load of 3.0 μg per well. The buffers used were running buffer (10 mM Tris base with glycine (3% w/v), sample buffer (glycerol (30% v/v) with running buffer (30% v/v) and bromophenol blue (about 0.01% w/v)), stacking buffer (470 mM Tris base pH adjusted to 6.7 with H_3PO_4), and separation buffer (1.5 M Tris base, pH adjusted to 8.9 with HCl). Visualization was done with Coomassie stain, and the gel was photographed (Canon EOS 400D).

SDS−PAGE of Insulin Reaction. The volumes of the reaction and control mixtures were reduced by half by vacuum centrifugation as in the case of the native PAGE experiments. The samples were then diluted 1:1 (v/v) with 10 mM ammonium acetate buffer (pH 7.4). Each sample (10 μL) was mixed with 3.0 μL of sample buffer: 63 mM Tris at pH 6.8, glycerol (10% v/v), SDS (2.5% w/v), and bromophenol blue (0.02% w/v). The samples were then boiled for 3 min before loading them on a 15% polyacrylamide gel with running buffer: 50 mM Tris, glycine (3% w/v), and SDS (0.1% w/v). Visualization was done with Coomassie stain, and the gel was photographed (Canon EOS 400D).

Enzymatic Digestion of Angiotensin I Reaction. Prior to digestion, the angiotensin I reaction and control mixtures were evaporated to dryness through vacuum-centrifugation (Savant SpeedVac, Techtum Lab, Umeå). Samples were then redissolved in each digestion buffer (see below) to a volume of 100−500 μL. For each experiment, three different enzymatic digestions were performed using trypsin, chymotrypsin, or proteinase K. Stock solutions of the proteases were 0.01 mg/mL trypsin in 50 mM ammonium bicarbonate (pH 8), 2 mg/mL chymotrypsin in 1 mM HCl and 2 mM $CaCl_2$, and 1 mg/mL proteinase K in 0.5 M ammonium acetate (pH 7.8) and 100 mM $CaCl_2$. The digestions were then run in the following way: tryptic digestion of angiotensin I (1:50 w/w) in 0.5 M ammonium acetate (pH 7.8) and 100 mM $CaCl_2$ at 37 °C for 24 h. Chymotryptic digestion of angiotensin I (1:50 w/w) in 0.5 M ammonium acetate (pH 7.8) and 100 mM $CaCl_2$ at 30 °C for 24 h. Proteinase K digestion of angiotensin I (1:20 w/w) in 10 mM ammonium acetate (pH 7.4) and 5 mM $CaCl_2$ at 37 °C for 18 h. Digested samples were desalted by ZipTip C18

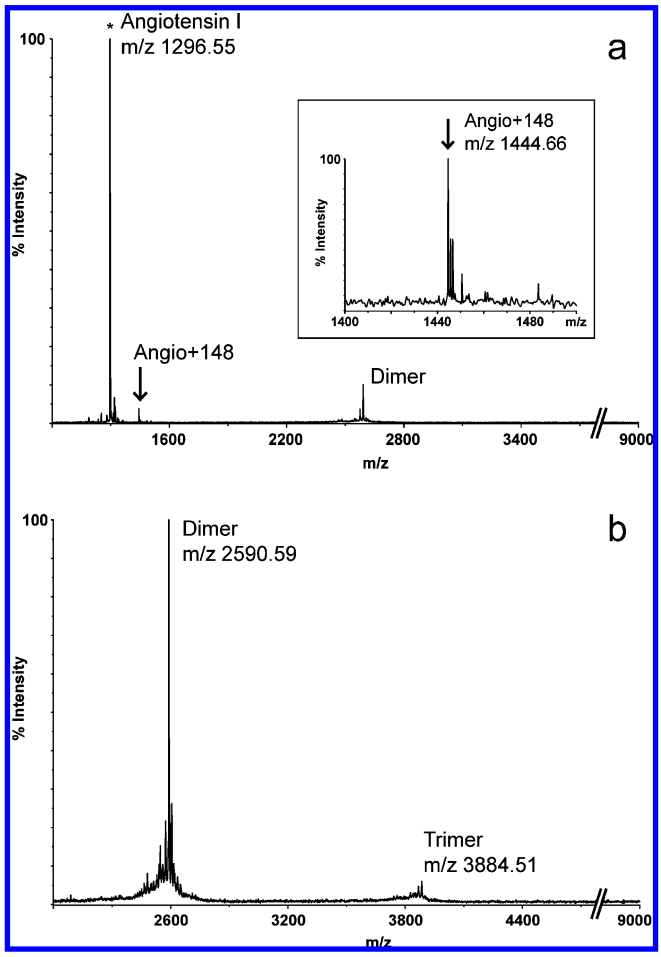

Figure 2. MALDI-TOF mass spectra of products from reactions between angiotensin I, Lim-2-OOH, and Fe(III)TPPCl (1:10:0.1 molar ratio) after 3 h of reaction time. Data were acquired at instrument settings of *m/z* range (a) 700−9000 or (b) 1400−9000. The sample in a was desalted by ZipTip C18 prior to analysis. The asterisk indicates unmodified angiotensin I at *m/z* 1296.55. Arrows indicate the angiotensin I+148 adduct at *m/z* 1444.66. The angiotensin I dimer was detected at *m/z* 2590.59 and angiotensin I trimer at (average) *m/z* 3884.51.

prior to MALDI/TOF-MS analysis, following instructions from the manufacturer. A matrix solution of 4 μL (saturated HCCA in 1:1 (v/v) ACN:0.1% TFA) was used to elute the material from the ZipTips, followed by 1 μL of 100% ACN to ensure maximum peptide recovery. MALDI/TOF-MS analysis was performed as described above.

Results

Control samples were prepared for every reaction mixture, excluding either hydroperoxide or Fe(III)TPPCl, or both. In the absence of Fe(III)TPPCl, no appreciable reaction of Lim-2-OOH was observed over 24 h (data not shown).

Stability of Lim-2-OOH in the Presence of Fe(III)TPPCl. The decomposition of Lim-2-OOH in the presence of Fe(III)TPPCl was explored by ^{1}H NMR. It showed that all Lim-2-OOH was converted into carvone (∼70%) and carveol (∼30%) within 100 min at 25 °C (see Supporting Information). Therefore, removal of unreacted hydroperoxide before peptide analysis in the reaction mixtures was determined to be unnecessary.

Angiotensin I-Lim-2-OOH Adducts. Of the three hydroperoxides, the only angiotensin I-hydroperoxide adduct detected was in the reaction mixture with Lim-2-OOH, at *m/z* 1444 (Figure 2a). From the peak areas in the MALDI spectra, this adduct was estimated to be formed at maximum 1% of the total amount of angiotensin I. No adducts could be detected with angiotensin I and CumOOH or CycHexOOH.

The angiotensin I-LimOOH adduct at *m/z* 1444 corresponds to the molecular weight of angiotensin I+148. The reaction mixture containing this adduct was subjected to LC/MS/MS to elucidate which amino acid(s) in the angiotensin I sequence had been modified. However, the amount of +148 adduct was too low to obtain fragmentation information. In order to increase the concentration of adduct for this analysis, attempts were made to improve the adduct yield by increasing the amount of Lim-2-OOH in the reaction mixture up to 1:100:0.1 (peptide/hydroperoxide/Fe(III)TPPCl), by varying the reaction temperature (37, 25, and 4 °C) and increasing the reaction time (5 h, 24 h, and 14 days). However, no increase in adduct amount was obtained. Therefore, the angiotensin I reaction mixture was digested by trypsin, chymotrypsin, and proteinase K, respectively, and then subjected to MALDI/TOF-MS. The MALDI/TOF data of these digestions are shown in Table 1.

Angiotensin I-Hydroperoxide Polymerization Products. A decrease in the amount of angiotensin I was observed for all three hydroperoxide reaction mixtures in the presence of iron porphyrin; however, it was not as pronounced as in the insulin reaction (described below). The $[M + H]^+$ ion of angiotensin I remained the main peak in the mass spectra from all reaction mixtures even after 3 h (Figure 2a). Typical results for all three hydroperoxide reactions are represented by the results from the experiments with Lim-2-OOH (Figure 2b), where singly charged peptide dimer and trimer peaks are observed at *m/z* 2590 and 3884, respectively. These molecular weights correspond to the loss of two hydrogens for each formed angiotensin−angiotensin bond. These oligomers were formed at detectable levels immediately after mixing the reagents (data not shown). No haptenated oligomers were observed in the reaction mixture. In the control samples of angiotensin I, neither adducts, polymers, nor reduction in peak intensity over time was detected.

Lim-2-OOH and Boc-Tyr-OMe or Bz-N-His-OMe Adducts. The amino acids tyrosine and histidine were individually reacted with Lim-2-OOH in the presence of Fe(III)TPPCl, and the reaction products were investigated for an amino acid +148 adduct by LC/MS. Both the amine and carboxyl ends of the amino acids were protected by derivatization to mimic their structure and reactivity as part of a protein chain. No +148 or other adducts were detected for the tyrosine, instead significant tyrosine dimerization was observed (data not shown). For histidine, a +148 adduct was detected at relatively high amounts in the presence of Fe(III)TPPCl, and this adduct was not detected at the histidine control samples. Mass spectra of unreacted histidine and the histidine +148 adduct are shown in Figure 3. Besides the +148 adduct, two peaks corresponding to histidine +150 were detected. The intensities of these peaks were approximately 10 times higher than that for the +148 adduct (data not shown).

Angiotensin I−Carvone Reaction Products. It has previously been shown that Lim-2-OOH is converted into the hapten carvone in the presence of iron porphyrin (*12*). Thus, the reaction pathway for the formation of the angiotensin I +148 adduct with Lim-2-OOH could possibly proceed directly from the formation of carvone. To determine if the angiotensin I +148 adduct is unique for Lim-2-OOH or if it is developed via carvone, a set of additional experiments with and without Fe(III)TPPCl were made in the presence of carvone instead of Lim-2-OOH. No adducts or polymers were detected, neither in the carvone reaction mixture nor in the control after 3 h at 37 °C (data not shown).

Table 1. MALDI-TOF-MS Analysis of Digested Products from the Reaction between Angiotensin I and Lim-2-OOH in the Presence of Fe(III)TPPCl[a]

enzyme	peptide sequence	theoretical $[M + H]^+$	detected $[M + H]^+$
trypsin	VYIHPFHL	1025.6	1025.5
	VYIHPFHL(+hapten)	1025.6 + 148.1 = 1173.7	1173.3
	VYIHPFHL(+hapten+O)	1025.6 + 148.1 + 16 = 1189.7	1189.3
	NR	289.2	288.3
	NRVYIHPF	1045.6	1046.5
chymotrypsin	NRVYIHPF	1045.6	1046.3
	NRVYIHPF(+hapten)	1045.6 + 148.1 = 1193.7	1194.5
	RVYIHPFH	1068.6	1068.4
	RVYIHP	784.4	784.4
	NRVY	551.3	552.2
	IHPF (or PFHL)	513.3	513.2
	PF	263.1	263.1
proteinase K	NRVYIHPF	1045.6	1046.6
	NRVYIHPF(+hapten)	1045.6 + 148.1 = 1193.7	1194.6
	NRVYIHPF(+hapten+O)	1045.6 + 148.1 + 16 = 1209.7	1210.6
	NRVY	551.3	552.3
	IHPF (or PFHL)	513.3	513.3
	HPF (or PFH)	400.2	401.1

[a] The full sequence of angiotensin I is NRVYIHPFHL.

Insulin−Hydroperoxide Reaction Products. Aliquots from the reaction mixtures of insulin with 100 molar excess of the three hydroperoxides were taken out at different times to monitor the product formation. Figure 4 shows the mass spectra immediately after mixing the reagents (Figure 4a, c, and e) and after 3 h (Figures 4b, d, and f). The arrows indicate one broad peak or clusters of just detectable peaks, about *m*/*z* 5860−5920, corresponding to the addition of one hydroperoxide molecule to the insulin. For all three hydroperoxides, the insulin signal was markedly decreased in the reaction mixtures over time compared to that in the control mixtures. After 3 h of reaction time, no or a very small insulin peak was detected using extensive sweet spot searching and high laser intensity (Figure 4b, d, and f). This means that only barely detectable amounts of unreacted insulin remained in the reaction mixture after 3 h. Furthermore, the signal corresponding to the mass of the insulin−hydroperoxide adducts decreased or disappeared after 3 h (Figure 4b, d, and f), which indicates that these products are consumed in a further reaction. As in the reactions with angiotensin I, neither adduct formation nor decrease in insulin signal was observed in any of the control samples. The reaction mixtures were also analyzed with MALDI/TOF-MS both at higher and lower *m*/*z* ranges, but no signals could be detected. There were no signs of either large molecular weight polymers or smaller degradation products.

Native PAGE was used to further investigate the insulin reaction products when they were still in oxidized and nondenatured forms (Figure 5). While distinctive bands of intact insulin were observed for all controls, the reaction mixtures (with both Fe(III)TPPCl and one of the three hydroperoxides) exhibited a continuous smear in their individual lanes, revealing an apparently broad range of high molecular weight products. Part of the sample in the reaction mixtures did not even enter past the stacking part of the gel (e.g., Figure 5, top of the lane with insulin + Fe(III)TPPCl + CumOOH). These insulin reaction products are likely aggregates and/or polymers which have molecular weights above 200 kDa.

The possible presence of covalently bound insulin oligomers was examined by subjecting reaction mixtures of Lim-2-OOH/insulin at molar ratios of 100:1, 10:1, and 1:1 to SDS−PAGE. The denaturing conditions of SDS−PAGE would dissociate any noncovalent insulin aggregate. Left to be separated on the gel were covalently bound species, with the exception of, e.g., disulfide bonds, as the reducing conditions would ensure that only covalent structures resistant to reduction remained. The only discrete protein bands distinguished on the gel corresponded to the comigration of the two intact and reduced monomer insulin α and β chains (Figure 6). Thus, the reaction products did not include any covalently bound insulin oligomers resistant to reduction. However, when both Lim-2-OOH and Fe(III)TPPCl were included in the reactions, a faint broadening of the insulin α−β-bands of up to approximately an addition of 3000 Da to the insulin chains could be seen in the lanes for all molar ratios. Consequently, in this case there were covalently modified insulin reaction products of a polydisperse nature which remained after both denaturation and reduction. MALDI/TOF-MS analyses of the same reduced samples did not provide any additional information since the individual species within the range of the modified insulin were at amounts below the detection limit.

Discussion

Three hydroperoxides, Lim-2-OOH, CumOOH, and CycHexOOH, were reacted in darkness with angiotensin I or the protein insulin using Fe(III)TPPCl as a radical initiator. The results showed that there are two possible routes of reactions: peptide−hapten binding and/or polymerization but that no reaction will take place without the addition of the radical initiator.

Angiotensin I. When angiotensin I was reacted with Lim-2-OOH in the presence of Fe(III)TPPCl, a small but distinctive peak at *m*/*z* 1444 was detected. This corresponds to the formation of a +148 monohaptenated adduct (Figure 2a). There were also peaks corresponding to dimers and trimers of angiotensin I (Figure 2b), but no haptenated dimer or trimer could be detected under the applied analytical conditions. The same relative amounts of angiotensin I dimers and trimers were also detected in the CycHexOOH and CumOOH reaction mixtures (data not shown). However, monohaptenated adducts could not be detected in the CycHexOOH and CumOOH reaction mixtures.

The amount of angiotensin I +148 adduct in the Lim-2-OOH experiment did not permit any structure determination by LC/MS/MS. Therefore, the angiotensin I reaction mixture was digested by three different enzymes to elucidate which amino acid had been modified. From the data in Table 1, it can be concluded that the hapten was bound somewhere in the sequence

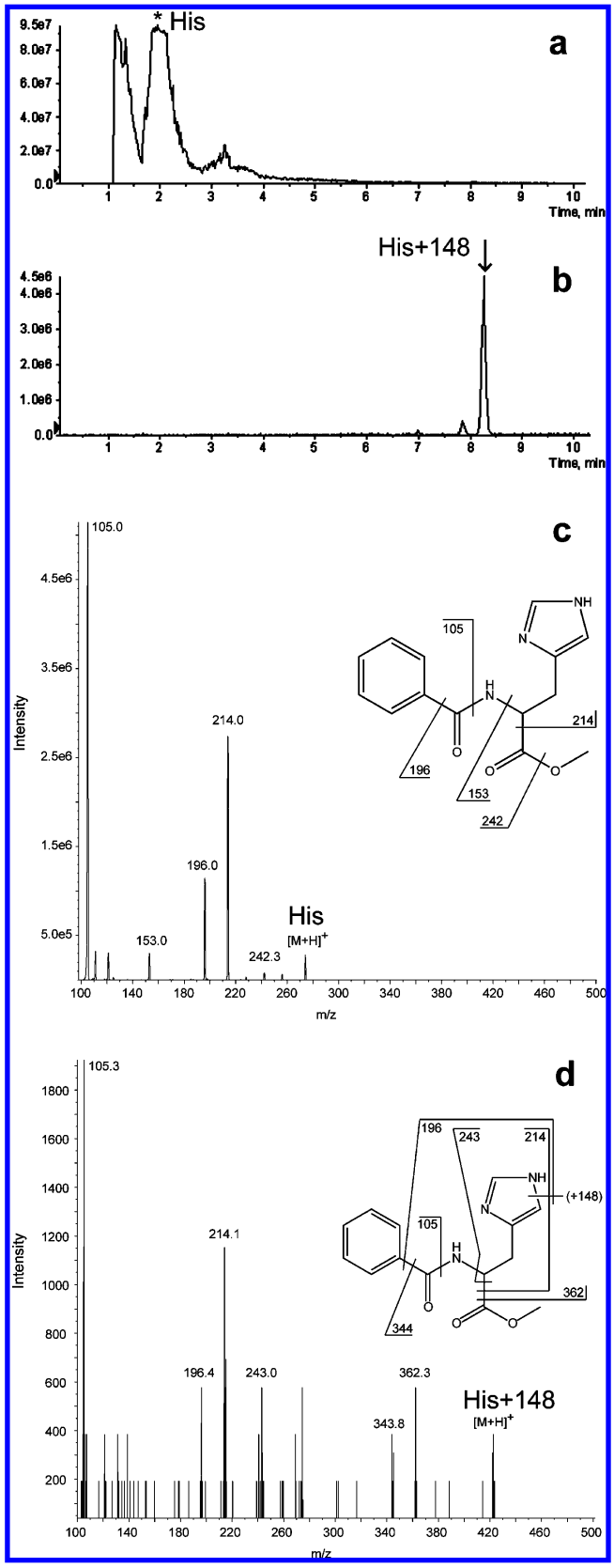

Figure 3. LC/MS analysis of histidine/Lim-2-OOH/Fe(III)TPPCl (1:10:0.1 molar ratio) reaction products. Extracted ion chromatograms of (a) unmodified His at *m/z* 274 which has not reacted (indicated by an asterisk) and (b) the His+148 adduct at *m/z* 422 (indicated by an arrow). Mass spectra after MS/MS fragmentation of (c) unreacted histidine (collision energy 30 V) and (d) the histidine +148 adduct (collision energy 40 V).

VYIHPF, of which only tyrosine and histidine were considered as likely candidates since pure hydrocarbon structures of the other amino acid side chains should not be reactive toward the Lim-2-OOH hapten. In the trypsin and proteinase K digestions, an oxidized hapten bound to the peptides was observed in the mass spectra (Table 1). These oxidized adducts (angiotensin I +148 +16) were not detected in the chymotrypsin digest or in the nondigested sample. Thus, they were probably formed during the trypsin and proteinase K digestions and not from the reaction between Lim-2-OOH and angiotensin I.

When tyrosine and histidine were separately subjected to reactions with Lim-2-OOH and any +148 adduct formation was examined by LC/MS/MS, only the histidine reaction mixture showed a positive result for a +148 adduct using LC/MS/MS (Figure 3b). The adduct formed exhibited a high stability toward MS/MS, requiring a collision energy as high as 40 V to fragment the quasi-molecular ion. The high stability could be due to a formed conjugated double bond within the structure. As shown in Figure 3d, the most intense fragments from the +148 adducts were the benzoyl ion (*m/z* 105) from the protected N-terminal, *m/z* 362 that corresponds to the neutral loss of the methyl ester

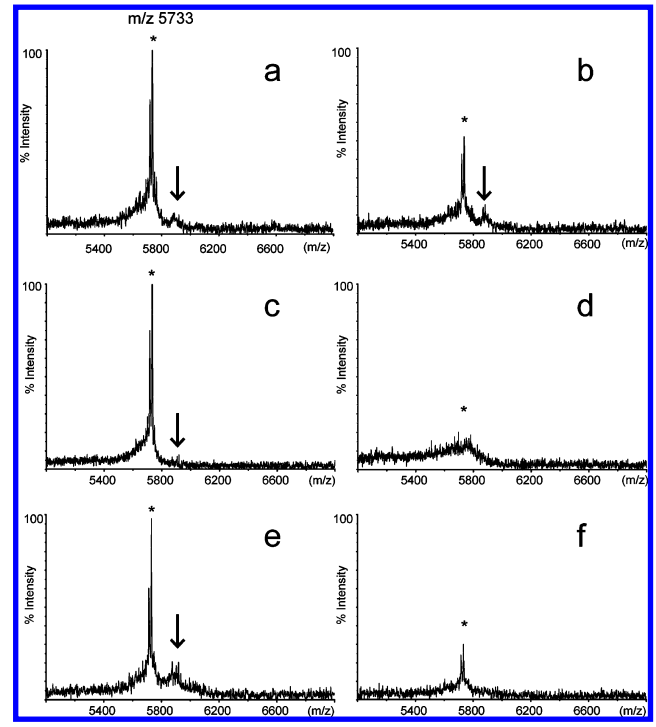

Figure 4. MALDI-TOF mass spectra of products from reactions among insulin, hydroperoxide, and Fe(III)TPPCl (1:100:0.1 molar ratio) using the hydroperoxides Lim-2-OOH (a,b), CumOOH (c,d) or or CycHexOOH (e,f). Samples were analyzed immediately after mixing (a, c, and e) and again after 3 h (b, d, and f). The asterisk indicates unmodified insulin at *m/z* 5733 (average). Arrows indicate one broad peak or clusters of several minor peaks, in the range of *m/z* 5860−5920.

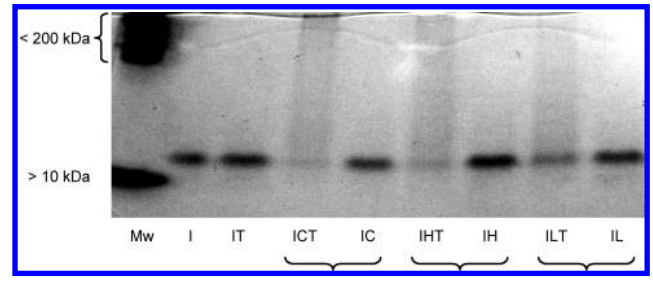

Figure 5. Native PAGE of insulin/hydroperoxide/Fe(III)TPPCl (1:10:0,1 molar ratio) reaction mixtures and control samples after 3 h of reaction. I = insulin, T = Fe(III)TPPCl, C = CumOOH, H = CycHexOOH, and L = Lim-2-OOH.

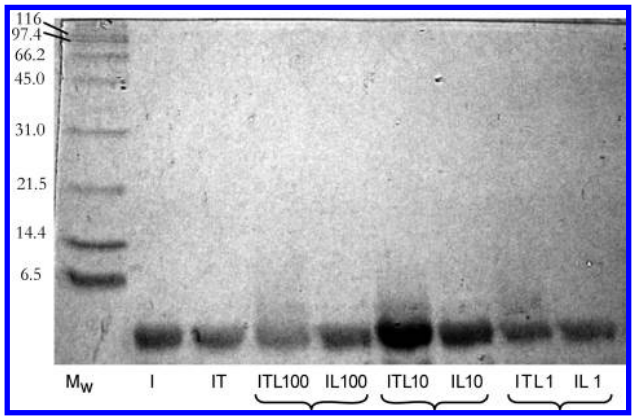

Figure 6. SDS−PAGE of insulin/Lim-2-OOH/Fe(III)TPPCl reaction mixtures at three different molar ratios of Lim-2-OOH, 1:(100, 10, or 1):0.1 after 3 h of reaction. I = insulin, T = Fe(III)TPPCl, and L = Lim-2-OOH. Control samples, without Fe(III)TPPCl (T) were preformed for every Lim-2-OOH concentration.

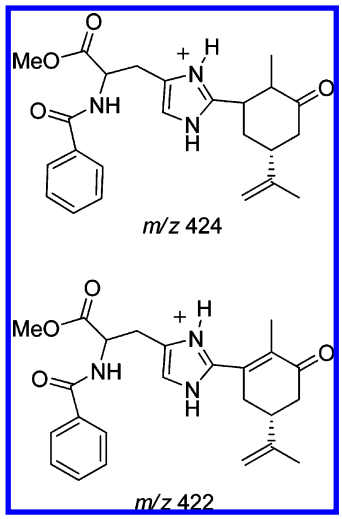

Figure 7. Possible structures of the $[M + H]^+$ quasi-molecular ions, *m/z* 424 and *m/z* 422, respectively, of the His+150 and His+148 species identified in the reactions of Lim-2-OOH with Fe(III)TPPCl in the presence Bz-N-His-OMe.

group (−60 Da from the C-terminal) and *m/z* 214, which is the histidine skeleton. The fragmentation pattern in the low mass region for the adduct is very similar to the spectrum of the unreacted histidine in Figure 3c, indicating that the histidine structure in the adduct is unchanged. It was also observed that the histidine +150 adducts required less collision energy for fragmentation than the +148 adduct. This indicates that the +148 adduct, which has lost two hydrogens compared to the +150 adduct, most likely has formed a conjugated double bond, located in the terpene moiety (Figure 7).

From previously conducted radical reaction experiments with protected cystein, only cystein adducts of molecular weights +150 were observed when Lim-2-OOH was reacted in the presence of Fe(III)TPPCl (*13*). These adducts correspond to the addition of a carvone moiety to cystein.

Angiotensin I has two histidine units in its sequence; it is interesting to note that all data obtained here show that only the first histidine (from the N-terminal end) is haptenated by Lim-2-OOH. This reactivity differs from what is observed for angiotensin I in the nucleophilic addition reaction with styrene oxide, where both histidines are equally reactive (*14*). This difference in reactivity maybe caused by a variation in the availability of the different histidines because of the dissimilar steric environment of the reacting histidine. The first histidine in angiotensin I is a bit more exposed due to the neighboring proline, while the second histidine is sterically hindered by the surrounding Phe and Leu side chains.

As the hydroperoxide had to be activated by Fe(III)TPPCl to participate in any reaction, the involvement of a radical mechanism was strongly suspected. To further investigate the importance of a radical reaction involvement, a reaction between angiotensin I and carvone (**4**, Figure 1) was studied. Carvone is the major decomposition product of Lim-2-OOH and a known hapten (*15*) which reacts with proteins through an electrophilic−nucleophilic Michael addition mechanism. No peptide−hapten adducts or polymers were detected in the reaction mixture of carvone or its control. This shows that the reaction mechanism for protein−hapten binding of Lim-2-OOH with angiotensin I is not achieved through a Michael addition reaction. Thus, there is a strong indication the angiotensin I +148 adduct and the histidine +148 adduct are formed through a radical pathway. Furthermore, the molecular weights of the dimer and trimer of angiotensin I (Figure 2) correspond to the loss of two hydrogens for each peptide−peptide bond formed rather than the loss of water. It is, therefore, likely that the polymers are also formed through a radical mechanism. This is supported by the observation that tyrosine forms dimers after exposure to hydrogen peroxides and lipid hydroperoxides (*16*). Interestingly, hemoglobin in red blood cells forms dimers and trimers through dityrosine binding when exposed to hydrogen peroxide, and similar to our results, these species are formed only in the presence of both hydrogen peroxide and the iron-containing heme group (*17*). In our experiment with protected tyrosine, significant dimerization was also observed.

Even though the amino acids involved in the binding of the observed angiotensin I polymers are currently unidentified, it is clear that the formation of the same molecular weight peptide polymers is independent of the hydroperoxide used.

We have in previous animal experiments investigated if cross-reactions between Lim-2-OOH, CycHexOOH, and CumOOH could be obtained but found no such reactions (*10*). We have also patch tested dermatitis patients with known contact allergy to one hydroperoxide with a structurally different hydroperoxide but found no increase in the number of positive reactions to this second hydroperoxide compared to that obtained by testing patients without contact allergy to hydroperoxides (*10*). In a subsequent study, we observed that (4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide was significantly more sensitizing compared to Lim-2-OOH in experimental studies and also gave more positive patch test reactions in dermatitis patients with known contact allergy to oxidized limonene containing both hydroperoxides (*18*). Thus, neither the human data nor the animal studies support the hypothesis that hydroperoxides form nonspecific antigens but rather indicate that hydroperoxides form specific immunogenic complexes in contact allergy. Therefore, the polymerization alone is unlikely to account for the hydroperoxide specificity in the hapten formation, and its role in developing ACD remains uncertain. Instead, the specificity of hydroperoxides must, therefore, be due to haptenated adducts such as angiotensin I +148 or similar, with most of the original hydroperoxide structure still attached to peptide or protein.

Insulin. To further test the reactivity of hydroperoxides and to test the possibility to form immunogenic complexes with proteins, the hydroperoxides were reacted with insulin in the presence of a radical initiator (Fe(III)TPPCl). Although, no adduct formation with CumOOH and CycHexOOH was observed for angiotensin I, peaks corresponding to the binding of

one hydroperoxide moiety to one insulin were detected for all three investigated hydroperoxides immediately after mixing (Figure 4a, c, and e). These data suggest that a unique hydroperoxide−insulin adduct is formed for each specific hydroperoxide. This initial adduct formation is very fast, and over time, the adducts disappear, and a substantial amount of polymerization of insulin is detected. The polymerization products formed with insulin could not be detected using MALDI/TOF-MS, indicating that the main products are highly polydisperse and/or difficult to ionize. This was confirmed by the native PAGE analysis (Figure 5), where in each reaction mixture containing both hydroperoxide and Fe(III)TPPCl, the original insulin band is lost in favor of a continuous smear. Evidently, insulin displays a range of large molecular weight reaction products. Insulin has the ability to form cytotoxic noncovalent aggregates (*19*) and as seen in Figure 6, most of the original insulin bands are recovered at reducing and denaturing conditions. Whether the large molecular weight insulin products are polymers and/or noncovalent aggregates has not been further investigated. However, most of the original α- and β-chains of insulin were recovered under the reducing conditions of the SDS−PAGE (vide infra), indicating that polymerization is in part caused by the formation of disulfide bridges.

Further indications of insulin modifications of up to an additional molecular weight of about 3000 Da for the α- and β-chains of insulin can be observed as weak smearing of the peptide bands in the SDS−PAGE reaction mixtures containing Lim-2-OOH and Fe(III)TPPCl (Figure 6). These modifications of the α- and β-chains would correspond to one or more Lim-2-OOH moieties. Analogous to angiotensin I, the histidines of the insulin β-chain could have been modified. There is also a possibility that the redox conditions of the models systems could generate free cystein or thiyl radicals that would react with Lim-2-OOH or its major degradation product carvone. In a previously conducted study, it was shown that cystein can be modified by Lim-2-OOH via a thiol−ene radical mechanism (*13*).

It should be noted that any adduct formed in these model systems does not necessarily correspond to the immunogenic complex formed in vivo. According to theory, the formation of a stable immunogenic complex is required to elicit an immune response. This is accomplished by the covalent binding of the hapten to a protein (*20, 21*). Even if the structures of the modified insulin are unknown, the results strongly suggest that Lim-2-OOH in the presence of a radical initiator can modify a protein and thus form a stable immunogenic complex that eventually results in an immune response.

One can speculate that the polymerization reaction is part of an overall radical reaction initiated by the presence of hydroperoxide and an initiator such as Fe(III)TPPCl. Large amounts of radicals are formed under these conditions (*12*). Elevated levels of radicals formed from hydroperoxides in the skin will increase the oxidative stress by the consumption of antioxidants (*22, 23*). Furthermore, the radical originating from hydroperoxides would increase the possibility of radical formation on macromolecules such as proteins. Reaction of protein radicals with molecular oxygen results in the formation of reactive oxygen species (ROS) that will deplete antioxidant reserves even further. Thus, the action of hydroperoxides in ACD results in increased oxidative stress due to the formation of high amounts of radicals, which would aid the progression of ACD.

Conclusions

We propose that the formation of specific immunogenic hapten−protein complexes from hydroperoxides can proceed via a radical reaction since hapten adducts were observed only in the presence of a radical initiator. It is thus reasonable to expect that intact hydroperoxide molecules can penetrate into the skin and remain there until activated, and then react via a radical mechanism. On the basis of the present observations and the results from a previous study (*10*) where no cross-reactivity between the investigated hydroperoxides in animal experiments and clinical studies was observed, we conclude that the specificity of the immunogenic response in ACD is caused by specific hapten−protein complexes formed from the hydroperoxides. In parallel to the specific reactions, a polymerization reaction was detected for angiotensin I and insulin for all hydroperoxides. The role of the polymerization reaction in ACD is presently unclear but may play an important role in the overall toxicity of hydroperoxides in the skin.

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Supporting Information Available: Reaction of Lim-2-OOH in the presence of Fe(III)TPPCl as followed by ^{1}H NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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