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Identification of the nitration site of insulin by peroxynitrite

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Abstract: Our previous investigation indicated that insulin can be nitrated by peroxynitrite *in vitro*. In this study, the preferential nitration site of the four tyrosine residues in insulin molecule was confirmed. Mononitrated and dinitrated insulins were purified by RP-HPLC. Following reduction of insulin disulfide bridges, Native-PAGE indicated that A-chain was preferentially nitrated. Combination of enzymatic digestion of mononitrated insulin with endoproteinase Glu-C, mass spectrometry confirmed that Tyr-A14 was the preferential nitration site when insulin was treated with peroxynitrite. Tyr-A19, maybe, was the next preferential nitration site. According to the crystal structure, Tyr-B26 between the two tyrosine residues in insulin B-chain was likely easier to be nitrated by peroxynitrite. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insulin; nitration; Native-PAGE; HPLC; mass spectrometry

INTRODUCTION

Peroxynitrite ($ONOO^-$), the product of superoxide and nitric oxide reaction, has been observed in many inflammatory conditions [1,2]. It has recently been implicated in the inactivation of many enzymes, and nitration of specific tyrosine residues is responsible for it [3]. Because both rodent and human islets are highly sensitive to peroxynitrite-induced damage [4], and peroxynitrite is found to be formed in islet β-cells of acutely diabetic NOD mice [5], insulin may be a potential target of peroxynitrite during conditions involving accelerated rates of oxygen radical and nitric oxide generation in pancreatic islet β-cells.

Abdel-Wahab *et al.* [6] have reported the glycation of insulin in the islets of langerhans of normal and diabetic animals, and nitration of insulin by tetranitromethane (TNM) was also reported [7–11]. Recently we reported insulin nitration by peroxynitrite *in vitro* [12]. Insulin nitration affected the receptor binding and hypoglycemic capacity, and the conformational changes after nitration may be involved in insulin–receptor interactions. The initial work by Sokolovsky *et al.* [7] indicated that only two of the four tyrosines in insulin reacted with TNM as determined by the increase in absorption at 428 nm or by amino acid analysis for 3-nitrotyrosine. Under mild treatment of insulin (bovine zinc insulin) with TNM, as reported by Morris *et al.* later [8], monomeric products containing nitrotyrosine residues could be obtained. They described the isolation of mononitrotyrosine and dinitrotyrosine insulin in which nitration had taken place primarily (but not exclusively) at Tyr-A14, Tyr-A14 and Tyr-A19, respectively. Both these derivatives exhibited high hormonal activity. Carpenter *et al.* [9] reported that an insulin derivative containing four nitrotyrosine residues could be obtained by treatment of insulin (zinc-free bovine insulin) with TNM, and it held 50% of hormonal activity. Therefore, it is of particular interest to identify the modification sites within this functionally important protein. In the present study, Native-PAGE, and a combination of specific enzymatic digestion, followed by mass spectrometry, was used to identify the preferential nitration sites by peroxynitrite within the insulin molecule.

MATERIALS AND METHODS

Reagents

Porcine zinc insulin (28 IU/mg) was obtained from Wanbang Pharm Inc. (Xuzhou, China). HPLC-grade acetonitrile was obtained from Fisher (Fair Lawn, New Jersey, USA). Sequencing grade trifluoroacetic acid (TFA), endoproteinase Glu-C (from *Staphylococcus aureus* V8, EC 3.4.21.19), Guanidine HCl, iodoacetamide (IAA) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma (St. Louis, MO, USA). Acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ammonium persulfate, Tris-base, glycine, dithiothreitol (DTT), and coomassie blue were obtained from Amersham Biosciences (Uppsala, Sweden). The water used in these experiments was purified using a Milli-Q System (Millipore, Bedford, MA, USA).

High Performance Liquid Chromatography

The Agilent 1100 HPLC system used was equipped with a diode array detector and a Rheodyne model 7725i injector valve with a 500 µl loop. Reversed-phase high performance liquid chromatography (RP-HPLC) columns used included a Jupiter C-18 analytical column (250 × 4.6 mm, 5 µm, 300 Å) (Dikma, Beijing, China), a hypersil C-18 semipreparation column (250 × 8 mm, 5 µm, 300 Å) (Elite, Dalian, China),

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and a hypersil C-8 analytical column (250 × 4.6 mm, 5 μm, 300 Å) (Elite, Dalian, China). The mobile phase consisted of solvent A: 0.1% (v/v) TFA in water, and solvent B: 100% acetonitrile. Elution profiles were monitored at 210, 275 (for protein detection), and 360 nm (for nitrotyrosine detection at acidic pH), and peptide peaks were collected by hand. Where rechromatography was required, samples were lyophilized and reconstituted in 0.1%TFA/water prior to HPLC separation.

Preparation of Nitrated Insulin

Peroxynitrite was synthesized in a quenched-flow reactor as described elsewhere [13], and excess H_2O_2 was removed by treatment with MnO_2. Peroxynitrite concentrations were determined spectrophotometrically at 302 nm in 1 mol/l NaOH ($\varepsilon = 1670$ l/mol/cm). Nitration of porcine insulin was performed as described previously [12]. After nitration, the pH were adjusted to 2 with TFA, and the samples were applied to the C-18 analytical column at a flow rate of 1.0 ml/min. Insulin and nitrated insulin were separated using a linear gradient of solvent B from 30 to 45% in 20 min. The HPLC method allowed determination of the percent nitration by comparison of peak areas. Nitrated insulin was prepared by mixing insulin aliquots (10 × 10 mg) with two- to five-fold molar excess of peroxynitrite. The mixture was applied to a C-18 semipreparative column at a flow rate of 1.5 ml/min. Insulin and nitrated insulin were separated using linear gradients of 30–32% solvent B over 5 min, followed by 32–36% over 20 min and 36–45% over 10 min. The obtained fractions were dried under vacuum.

Separation of Insulin A- and B-Chains

The disulfide bridges of control and mononitrated insulins (100 μg) were reduced using DTT (5 mmol/l) in the presence of 6 mol/l guanidine HCl in a 100 mmol/l Tris–HCl buffer (pH 8.5, 200 μl) for 2 h at room temperature without alkylation. The insulin A- and B-chains were separated on a C-8 analytical column at a flow rate of 1.0 ml/min. Peptides were eluted using linear gradients of 30–33% solvent B for over 5 min, followed by 33–35% for over 20 min, 35–40% for over 20 min and 40–45% for over 5 min and collected manually.

In the meantime, Native-PAGE was applied to the analysis of the reduction products of insulin and nitrated insulin. Here samples were reduced with 5 mmol/l DTT at 50 °C for 15 min. After cooling to room temperature, the reduced samples were alkylated with 10 mmol/l freshly dissolved IAA in dark at room temperature for 30 min. A Hoefer miniVE vertical electrophoresis system was used with a discontinuous Tris–glycine gel. Separating gel and stacking gel consisted of 20 and 5% acrylamide, respectively. Upon completion of the electrophoresis, the gel was stained with Coomassie brilliant blue G-250.

Endoproteinase Glu-C

Mononitrated insulin (100 μg) was reconstituted in 100 μl of 100 mmol/l sodium phosphate buffer, pH 7.8, treated with endoproteinase Glu-C (10 μg) to specially hydrolyze the A- and B-chain at the *C*-terminal end of Glu (A4, A17, B13 and B21). The molecular weights of fragments were determined by mass spectrometry.

Matrix Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

Samples of lyophilized powder were dissolved directly in a matrix solution containing of 50% (v/v) acetonitrile/0.1% TFA saturated with CHCA, and the samples of digestion were desalted using C18 ZipTips (Millipore, Bedford, MA) and eluted directly onto the stainless steel sample plate with the matrix solution. Mass spectra were acquired on a Bruker Autoflex TOF mass spectrometer equipped with a 337-nm nitrogen laser. The instrument was operated in the positive linear (flight distance 1.22 m, accelerating voltage 20 kV) or reflector (flight distance 2.7 m, accelerating voltage 20 kV and reflection voltage 23 kV) mode.

RESULTS

HPLC Separation of Nitrated Insulin

Nitration of porcine insulin in phosphate buffer (pH 7.4) was dose-dependent on peroxynitrite [12], and it could also be confirmed by HPLC method. A typical HPLC chromatographic separation of insulin and nitrated insulin on a C-18 analytical column following treated insulin with five-fold molar excess of peroxynitrite is shown in Figure 1. Nitrated insulins were eluted behind insulin because the formation of nitrotyrosine may lead to a lower isoelectric point [12]. Indeed, nitration of tyrosine residues in proteins induces the change of tyrosine into a negatively charged hydrophilic nitrotyrosine moiety and causes a marked shift of the local pK_a of the hydroxyl group from 10.07 in tyrosine to 7.50 in nitrotyrosine [1]. As a result, in acidic mobile phase TFA pairs with the nitrated insulins resulting in relatively longer retention. The peak eluted at 7.8 min had a mass signal of 5778.6 Da [$(M + H)^+$], corresponding to native insulin (theoretical average 5777.7 Da). The second peak eluted at 9.7 min had a mass signal of 5823.7 Da (Figure 2), indicating the addition of one nitro group (45 Da) to insulin the molecule. Moreover, the ions at m/z 5807.9 and 5792.0 were also detected, in agreement with the fragmentation pattern observed when nitrated molecules were analyzed by MALDI, and these unique series of ions, which correspond to the addition of one nitro group and the loss of one and two oxygens, have become the characteristic of nitrated peptides [14,15]. However, a small shoulder (indicated by an arrow) was observed on the mononitrated insulin peak which can convert to two overlapped peaks when slowing eluent gradient change, and we concluded that it was the B3 deamido mononitrated insulin [16]. After a mononitrated insulin peak, there were dinitrated and trinitrated insulin peaks that had been split into two peaks, and one of which was deamido nitrated insulin. Using a C-18 semipreparative column, purified mononitrated insulin and dinitrated insulin were prepared by collecting the sharp peak elutent of

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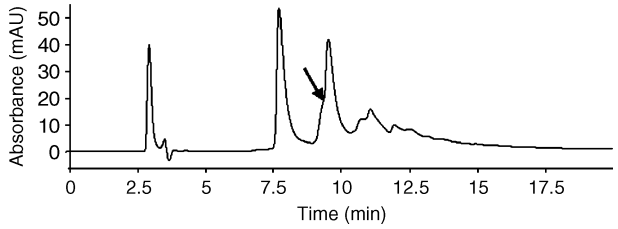

Figure 1 HPLC separation of insulin and nitrated insulin. Insulin (200 μmol/l) was treated with 5-fold molar excess of peroxynitrite, and the mixture was separated by RP-HPLC using a Jupiter C-18 column as described in the text. The chromatograph was the spectra at 275 nm, and the absorbance of 210 and 360 nm were similar.

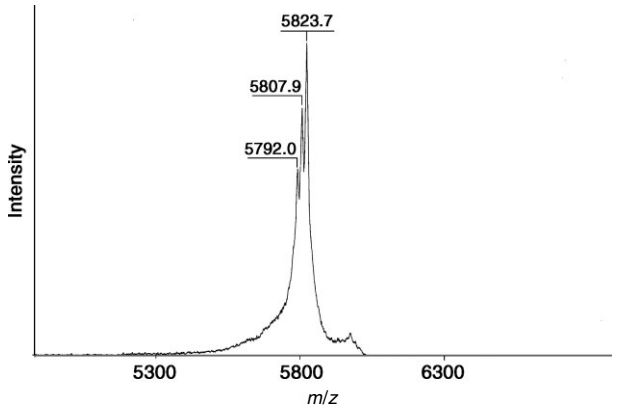

Figure 2 Linear MALDI spectra of mononitrated insulin purified by HPLC. The $(M+H)^+$ ion at m/z 5823.7 was corresponding to the nitration (+45 Da) of insulin (theoretical 5777.7) and associated ions at m/z 5807.9 (−16 Da) and 5792.0 (−32 Da).

mononitrated insulin and the strong peak between the two split peaks of dinitrated insulin.

Separation of Insulin A- and B-Chains

Purified mononitrated insulin and dinitrated insulin by HPLC were further confirmed by Native-PAGE (lanes 3 and 4 in Figure 3). Following the reduction of disulfide bridges by treatment with DTT, two methods were applied to separate the A- and B-chains of insulin and nitrated insulin. In Native-PAGE, the electrophoretic mobilities of peptides are primarily determined by the charges of their own, while the differences of molecular weight can be neglected. The migration mobility of insulin A-chain (pI 3.79) was greater than that of insulin B-chain (pI 6.90) because of the more negative charges of insulin A-chain in gels of pH 8.3. Nitration can increase the negative charges of peptides, and so compared with A- and B-chains of insulin (lane 5) in the gel, the peroxynitrite-treated insulin has two more bands below the A- and B-chains, respectively (lane 6), which correspond to the mononitrated and dinitrated A- and B-chains. The A-chain position of

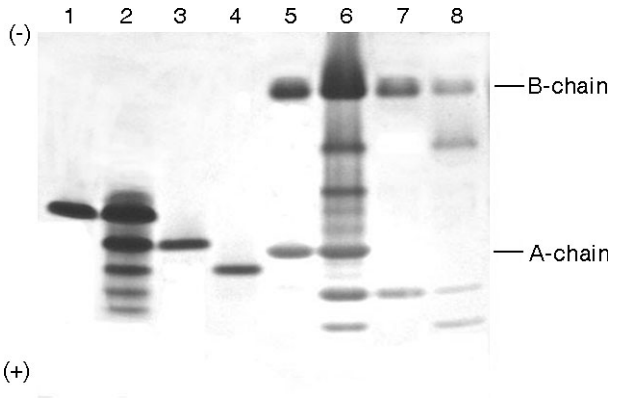

Figure 3 Native-PAGE of insulin and nitrated insulin. Samples were prepared as described in the text and subjected to a 20% polyacrylamide gel. lane 1, insulin; lane 2, insulin treated with 5-fold molar excess of peroxynitrite; lane 3, mononitrated insulin; lane 4, dinitrated insulin; lane 5, insulin treated with DTT and IAA; lane 6, insulin treated with 5-fold molar excess of peroxynitrite and followed by DTT and IAA; lane 7, mononitrated insulin treated with DTT and IAA; lane 8, dinitrated insulin treated with DTT and IAA.

mononitrated insulin in the gel indicated that the A-chain was preferentially nitrated when was treated insulin with peroxynitrite (lane 7), and dinitrated insulin included two main species (lane 8): one was that two tyrosine residues of A-chain were nitrated, and the other was that one tyrosine residue of A-chain and one tyrosine residue of B-chain were nitrated. In RP-HPLC, without IAA alkylation, the A- and B-chains of mononitrated insulin were collected and analyzed by MALDI-TOF-MS. Most (approx. 98%) of intact insulin was reduced to its component A- and B-chains under these experimental conditions. For insulin, the A-chain eluted at 13.6 min followed by the B-chain at 18.4 min (Figure 4(A)). For mononitrated insulin the A-chain eluted far behind the B-chain at 35.6 min (Figure 4(B)), and the molecular mass was measured as 2427.0 Da (theoretical monoisotopic 2382.0 Da) (Figure 5), indicating that a nitro group was added to insulin A-chain, and the ions corresponding to the loss of one and two oxygens were also observed.

Identification of Preferential Nitration Sites

The signal of insulin A-chain in MALDI-TOF-MS is very weak in the positive mode, and in negative mode it gets a little stronger; so it is difficult to perform MS/MS analysis to further analyze the nitration site in A-chain. Treated with the endoproteinase Glu-C, insulin could hydrolyzed to four fragments, A(1–4), A(5–17) + B(1–13), A (18–20) + B(14–21), and B(22–30), and because of containing parts of B-chain the two fragments containing parts of A-chain were easy to be ionized. The digest of mononitrated insulin were subjected to MS analysis (Figure 6), and the molecular masses of the four peptide fragments are shown

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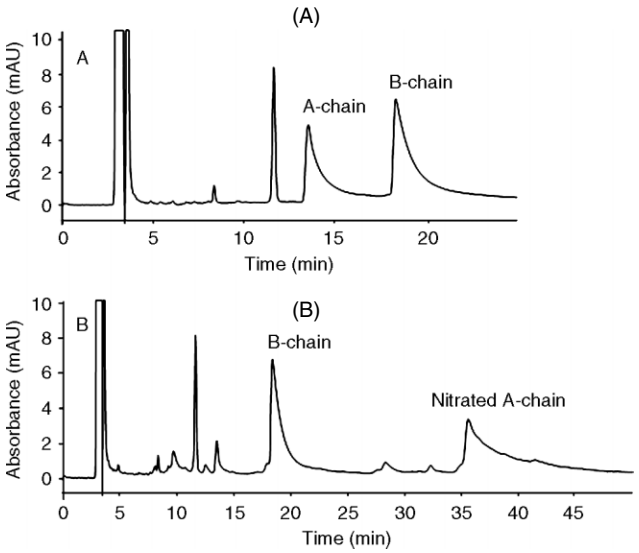

Figure 4 HPLC separation of A- and B-chains of insulin (A) and mononitrated insulin (B). Samples were prepared and separated using a Hypersil C-8 column as described in the text. Retention times: insulin A-chain, 13.6 min; insulin B-chain, 18.4 min; mononitrated insulin A-chain, 35.6 min, mononitrated insulin B-chain, 18.4 min.

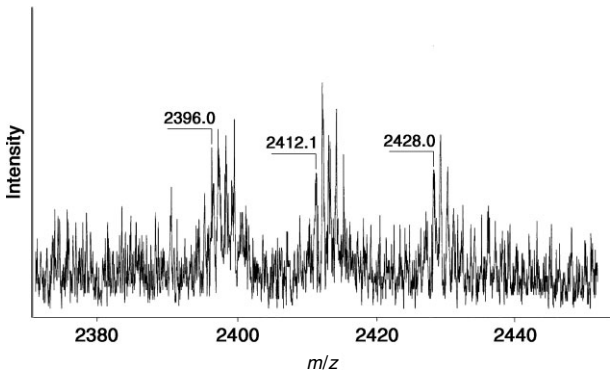

Figure 5 Reflector MALDI spectra of mononitrated insulin A-chain purified by HPLC. The $(M+H)^+$ ion at m/z 2428.0 was corresponding to the nitration (+45 Da) of insulin A-chain (theoretical 2382.0) and associated ions at m/z 2412.1 (−16 Da) and 2396.0 (−32 Da).

in Table 1. The ion at m/z 3013.5 $[(M+H)^+]$ was corresponding to the nitrated fragment of A(5–17) + B(1–13) (theoretical monoisotopic 2967.3 Da), so A14-Tyr was the preferential nitration site when insulin was treated with peroxynitrite. The peptide fragments of B-chain after breakage of the disulfide bridges were also observed, and the ion at m/z 1482.9 corresponded to B(1–13) (theoretical monoisotopic 1481.7 Da). The ions at m/z 1993.0 and 2347.3 were unknown, maybe corresponding to B(14–30) and B(1–21) in which some other modification had taken place. From the band color in lane 8, we can roughly conclude that Tyr-A19 was easier to be nitrated than the tyrosine residues of B-chain.

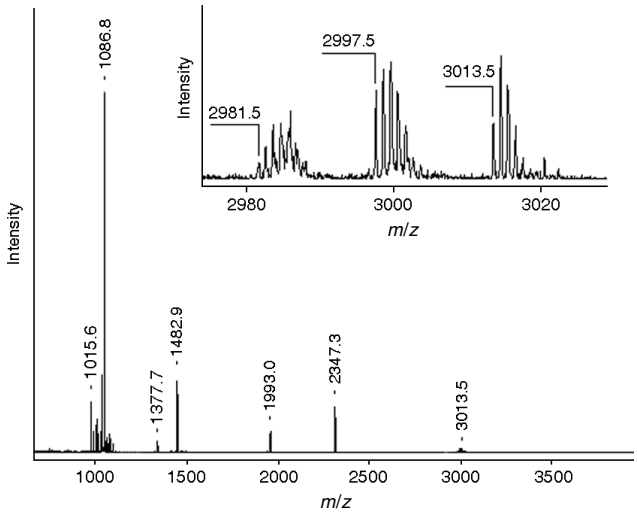

Figure 6 Reflector MALDI spectra of mononitrated insulin by endoproteinase Glu-c. The $(M+H)^+$ ion at m/z 3013.5 corresponds to the nitration (+45 Da) of the corresponding fragment A(5–17) + B(1–13) of insulin (theoretical 2967.3) and associated ions at m/z 2997.5 (−16 Da) and 2981.5 (−32 Da).

Table 1 Four peptide fragments of mononitrated insulin by Endoproteinase Glu-C

Fragments	Measured mass (Da) Monoisotopic $(M+H)^+$	Theoretical mass (Da) Monoisotopic
A(1–4)	Not detected	416.2
A(5–17) + B(1–13)	3013.5	2967.3
A(18–20) + B(14–21)	1377.7	1376.6
B (22–30)	1086.8	1085.6

DISCUSSION

The preferential nitration order confirmed the model of the insulin tyrosyl residues developed earlier [8]: (1) Tyr-A14 is exposed and hydrogen-bonded to a carboxylate group; (2) Tyr-A19 is at least partly exposed; (3) Tyr-B16 and B26 are not exposed, at least in aggregated forms of insulin. The crystallographic structure [17] confirmed the above model and showed that Tyr-A14 can form a hydrogen bond with the carboxylate group of Glu-A17. Ischiropolous [18] reasoned that the negative charge of glutamate provides a local environment for directing nitration to the neighboring tyrosine residues. In this structure, the *N*-terminus of the B-chain (Bl–8) is in an extended conformation, residues B9–19 are α-helical, residues B20–22 are involved in a β-turn, and B23–30 form a β-strand that interacts with the central helix to complete the characteristic insulin fold (T-state). Because of the mobility of the β-strand of B23–30, we conclude

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that Tyr-B26 may be more active than Tyr-B16 when nitrated by peroxynitrite.

Recent investigations indicated that the surface exposure of the tyrosine residue, the absence of steric hindrance and the proximity to a negatively charged residue constituted the structural requirements for the selectivity of protein tyrosine nitration [18]. Selective nitration of Tyr-A14 of insulin by peroxynitrite provides another evidence for these. Nitration of proteins can alter their biological activity, and previous reports have shown that nitrated insulin exhibits decreased hypoglycemic activity [12]. These observations may promote further studies to demonstrate insulin nitration and determine the site(s) of insulin nitration *in vivo* to establish if these differ from the *in vitro* situation.

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