Two new elastin cross-links having pyridine skeleton: Implication of ammonia in elastin cross-linking \textit{in vivo}.

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Running Title: Desmopyridine and isodesmopyridine in elastin
SUMMARY

Isolation and structure analysis of two amino acids from bovine ligamentum nuchae elastin hydrolysates revealed the presence of pyridine cross-links in elastin. The structures of these amino acids were determined to have 3,4,5- and 2,3,5-trisubstituted pyridine skeletons both with three carboxylic acids and a mass of 396 (C_{18}H_{28}N_{4}O_{6}), identified as 4-(4-amino-4-carboxybutyl)-3,5-di-(3-amino-3-carboxypropyl)-pyridine and 2-(4-amino-4-carboxybutyl)-3,5-di-(3-amino-3-carboxypropyl)-pyridine. We have named these pyridine cross-links, desmopyridine (DESP) and isodesmopyridine (IDP), respectively. Structure analysis of these pyridine cross-links implied that the formation of these cross-links involved the condensation reaction between ammonia and allysine. The elastin incubated with ammonium chloride showed DESP and IDP levels increased as the allysine content decreased. DESP and IDP were measured by HPLC with UV detection and were found in a variety of bovine tissues. The DESP/desmosine and IDP/isodesmosine ratios in aorta elastin were higher than in other tissues. DESP and IDP contents in human aorta elastin were found to be gradually increased with age. The concentration of IDP was significantly elevated in aorta elastin of rat with chronic liver cirrhosis induced by carbon tetrachloride (mean ± S.D.; 11.1 ± 0.9 nmol/mg elastin) when compared with normal rats (5.9 ± 1.5 nmol/mg elastin). Although DESP and IDP are present at only trace concentrations in the tissue elastin, these pyridine cross-links may be useful biomarkers for the aortic elastin damaged by ammonia.

Key Words: allysine; ammonia; aorta; cross-link; desmosine; desmopyridine; elastin; isodesmosine; isodesmopyridine.
INTRODUCTION

The elastic properties of several vertebrate tissues such as lung, skin, and large blood vessels are mainly due to the presence of elastic fibers. Elastin, the major component of these fibers, is synthesized by mesenchymal cells as a soluble precursor, tropoelastin, which undergoes post-translational modifications leading to the formation of specific cross-links that join together several tropoelastin chains (1).

All of the cross-links in elastin are formed spontaneously after oxidative deamination of specific lysine residues of tropoelastin by lysyl oxidase in the extracellular space. Formed reactive aldehyde, allysine, react with lysine and/or another allysine to form polyfunctional cross-links such as allysine-aldol, lysinonorleucine, merodesmosine, desmosine (DES), isodesmosine (IDE), and cyclopentenosine (2-10).

Elastin and collagen are the key structural components of the mammalian artery wall, particularly in the aorta (11). In concert with other extracellular matrix macromolecules, these two proteins provide the structural integrity and resiliency required of this specialized blood vessel (12). Extracellular matrix proteins, such as collagen and elastin, are known to be greatly affected by age because of impaired functional properties and increased susceptibility to diseases (13). Collagen undergoes progressive changes that are characterized by decreased solubility, decreased proteolytic digestibility, increased heat denaturation time, and accumulation of yellow and fluorescent material (14-16). These changes are thought to result from the formation of age-related intermolecular cross-links (17, 18). Also, age-related changes of elastin are its fragmentation and progressive proteolysis (19). These changes are accompanied by reduction of elastin in itself and content of elastin cross-links. Previously, we isolated two new dihydrooxopyridine cross-links, oxodesmosine (OXD) and isooxodesmosine (IOXD) from the acid hydrolysates of the bovine aortic elastin (20). Recently, we found that OXD and IOXD are oxidative metabolic intermediates generated from DES and IDE in vitro, respectively, by reactive oxygen species (21). Structures of DES, IDE, OXD, and IOXD are shown in FIG.7. Little is known about the formation of cross-links associated with the damaged elastin in vivo.

This study was undertaken to elucidate the chemical nature of cross-linking structures of elastin initiated by lysyl oxidase. We isolated two pyridine cross-links, desmopyridine (DESP) and isodesmopyridine (IDP) from ligamentum nucahe elastin hydrolysates. Using a reverse-phase HPLC assay, we measured the levels of these pyridine cross-links in bovine tissue elastin and showed that the concentrations of both cross-links increased in
concert with age in human aorta elastin. In the present study, we found that DESP and IDP were formed during incubation of ammonium chloride with ligamentum nuchae elastin consistent with the decrease of allysine residues. Compared to normal rats, the IDP concentration was found to increase in rat aorta elastin with liver cirrhosis induced by carbon tetrachloride, consistent with the decrease of allysine. These results suggested that allysine residues undergo a Chichibabine reaction (22, 23) with ammonia to form DESP and IDP in elastin. IDP may be an abnormal cross-link in elastin.
EXPERIMENTAL PROCEDURES

Materials

Acetonitrile (HPLC grade), p-cresol, sodium dihydrogen phosphate, hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), carbon tetrachloride, mineral oil (all analytical grade), di-phosphorous pentaoxide (P₂O₅) were purchased from Nakalai Tesque (Kyoto, Japan). Activated charcoal (60-150 mesh) and Silica gel 60 (70-230 mesh) for column chromatography were obtained from Nacalai Tesque, and silica gel (Art. 7734) for column chromatography from Merck (Germany). Deuterium dioxide (D₂O) and dioxane D₈ for NMR spectroscopy were obtained from EURISO-TOP (France).

Animal Experiments

Male Wistar rats (200-230 g, 8 weeks of age) were used in the experiments. The animal studies were in strict accordance with the guidelines established by the Canadian Council on Animal Care. Chronic liver injury was induced in rats by repeated intraperitoneal injections of carbon tetrachloride/mineral oil (1:1) at a dose of 1 ml/kg of body weight three times a week (on Monday, Wednesday, and Friday) for a total of 10 injections (24). Rats were killed under diethyl ether anesthesia 4 days after the last injection. Rats were used for this study only when evidence of cirrhosis was confirmed histologically.

Preparation of the Elastin

The elastin from various bovine tissues was purified by the modified technique with 1M NaCl treatment described previously (25) as follows. After removing peripheral connective tissue and lipids, bovine tissues (wet weight 10g) cut into small segments with a mixer were suspended in 1M NaCl (50ml) for 24hr. The supernatant was discarded after centrifugation, and then the pellet was re-suspended in 1M NaCl. This extraction process was repeated three times. The insoluble residue was washed with distilled water. After centrifugation, the insoluble residue was washed with excess of distilled water and defatted with chloroform/methanol (2:1, v/v) for 48hr, being washed with ethanol and ether. The prepared elastin was dried in vacuo under P₂O₅. The human aorta was kindly supplied by the Department of Pathology, Tohoku University Hospital. Human aortic samples were obtained from 11 autopsy cases which had no particular aortic diseases but had sclerotic changes corresponding with their ages. The range of age was from 19 to 75 year-old. The materials were taken from non-atherosclerotic
areas of thoracic aorta, and were preserved in methanol at -20°C until analysis. Human aorta elastin was purified by the technique as described above.

Isolation of DESP and IDP from Acid Hydrolysates of Elastin

The elastin for the isolation of DESP and IDP was prepared from fresh bovine ligamentum nuchae (about 1 kg). After acid hydrolysis of elastin with 6N HCl (110°C, 48 h), an aliquot of 30 g of its acid hydrolysates was dissolved in distilled water (40ml), and then charged on a large scale charcoal column (180×65 mm). Major lysine derived cross-linking amino acids were fractionated with water (3L) followed by elution with 20% aqueous methanol solution (3L). The 20% aqueous methanol fraction was evaporated at 50°C. The residue was dissolved with 40 ml of ethyl acetate/acetic acid/water (1.5:1:1, v/v), and then charged on a large scale silica gel column (130×65 mm). After the elution of neutral amino acids with a solvent (1.5 L) of ethyl acetate/acetic acid/water (1.5:1:1, v/v), most of the lysine-derived cross-links were eluted with water (0.5L). The water elution was evaporated at 50°C. The residue was dissolved with 40 ml of ethyl acetate/acetic acid/water (1.5:1:1, v/v), and then an aliquot of 2ml charged on a LiChroprep Si60 (40-63µm) preparative silica gel column (310-25 mm, Merck, Darmstadt, Germany) and fractioned using ethyl acetate/acetic acid/water (1.5:1:1, v/v) as a solvent, at a flow rat of 3.0 ml/min. DESP and IDP were eluted at 160-180 min. and 190-210 min., respectively. After removing the solvent by evaporation at 50°C, the residue containing DESP or IDP was dissolved with water (1.0 ml) containing 0.02 N HCl, and then charged on a preparative ODS column (40-63µm) (LiChroprep RP-18, 310-25 mm, Merck, Darmstadt, Germany). Both DESP and IDP were eluted at 20-24 min using a solvent of water (1.0 ml) containing 0.02 N HCl, at a flow rate of 3.0 ml/min. After 5 times repeat of this method, DESP and IDP were purified by re-chromatography with a preparative silica gel column, respectively. The purity of DESP and IDP was confirmed by silica gel TLC and analytical HPLC. TLC was conducted by pre-coated Kieselgel 60 on an aluminum sheet (Merck, Germany) using ethyl acetate/acetic acid/water (2:1:1, v/v) as a solvent. DESP and IDP were eluted just before DES and IDE revealed characteristic strong ninhydrin-positive spot with both Rf of 0.08 (DES, IDE; origin) on TLC and elution before IDE with Rf of 20.2 and 19.0 min (DES and IDE; Rf of 34.7 and 24.7 min, respectively) on analytical HPLC.

DESP and IDP aren’t artifacts formed from DES and IDE, respectively, by acid hydrolysis. In order to elucidate this problem, prolong hydrolysis of elastin or pure DES and IDE were carried out under the condition
of 110°C for 240 h in 6N HCl. DES and IDE were isolated from acid hydrolysates of bovine ligamentum nuchae elastin by the method of Nakamura and Suyama (26).

**In Vitro Incubation of Elastin with Ammonium Chloride**

Dried elastin powder (50 mg) was incubated with 1.0 M ammonium chloride in 5 ml of 0.2 M phosphate buffer (pH 7.4) containing 10 µl each of toluene and chloroform to prevent microbial growth. As a control experiment, dried elastin powder (50 mg) was incubated without ammonium chloride in 5 ml of 0.2 M phosphate buffer (pH 7.4) containing 10 µl each of toluene and chloroform to prevent microbial growth. The incubation was carried out at 37°C (or 60°C) for the time indicated in FIG. 8. After incubation, insoluble elastin was washed free of excess ammonium chloride three times with distilled water, and then twice with ether/ethanol. The resulting precipitate was dried *in vacuo* under P₂O₅ overnight, and an aliquot of 25 mg was subjected to acid hydrolysis with 6N HCl for 48 h at 110°C.

**Determination of DESP and IDP by HPLC**

Elastin hydrolysates were evaporated to dryness *in vacuo* at 50°C. The residues was dried over P₂O₅ and diluted with water (1.0 ml), and then a 20µl portion of solutions was injected onto the reverse-phase HPLC column. The HPLC system consisted of a SHIMADZU (Kyoto, Japan) LC-6A pump, a SPD-6AV UV-VIS spectrophotometric detector, a C-R5A data station, and a HITACHI (Tokyo, Japan) L-7300 column oven. Separation was performed with a Mightysil RP-18 150-4.6 (5 µm) reverse-phase (Kanto Chemical, Tokyo, Japan). The flow-rate was 1.0 ml/min. All HPLC chromatographic operations were carried out at 40°C. Determination of pyridine crosslinks was performed with analytical HPLC using a solvent of 0.1 M phosphate buffer-acetonitrile (5:1, v/v) containing 20 mM SDS (at pH 3.6). Concentrations of DESP and IDP were determined by comparison with DES and IDE, respectively, using analytical HPLC and monitoring at 275 nm.

**Determination of Allysine as p-Cresol Derivative by HPLC**

The allysine concentrations in elastin was determined as APC, 2-amino-6,6-bis(2-hydroxy-5-methylphenyl)hexanoic acid, as described previously (27). Derivatization of allysine in elastin was carried out under condition of acid hydrolysis (6N HCl, 110°C, 48 h). Twenty five milligrams of elastin was precisely
weighed and then dissolved in 5 ml of 6N HCl containing 5 % (w/v) p-cresol in a flame-sealed Pyrex glass. Derivatization of allysine in elastin was carried out under the condition of acid hydrolysis at 110°C for 48 h. Five milliliters of n-hexane/ether (8:2, v/v) was added to the solution after the reaction, and then the organic layer was discarded. Excess p-cresol was extracted with n-hexane/ether (8:2, v/v). APC was not detected by HPLC in the organic layer. The resulting solution was evaporated to dryness in vacuo at 50°C. The residue was dried over P₂O₅ and dissolved with HPLC solvent (10 ml); then a 20µl portion of solution was injected into the reverse-phase HPLC column. The HPLC system consisted of a SHIMADZU (Kyoto, Japan) LC-6A pump, a SPD-6AV UV-VIS spectrophotometric detector, a C-R5A data station, and a HITACHI (Tokyo, Japan) L-7300 column oven. Separation was performed with a LiChrosper 100 RP-18 125-4 reverse-phase column (Merck, Darmstadt, Germany). A guard column (LiChrosper 100 RP-18, 4×4 mm I.D., 5µm particle size; Merck, Darmstadt, Germany) was placed just before the inlet of the analytical column to reduce contamination of the analytical column. The mobile phase was composed of 0.05 M sodium dihydrogen phosphate (pH2.2)-acetonitrile (3:1). The flow-rate was 1.0 ml/min and the column temperature was 30°C. The UV signal was monitored at 282 nm which corresponds to an absorption maximum for APC.

**Determination of Allysine-aldol by HPLC**

Allysine-aldol was determined as the pyridine derivative, 6-(3-pyridyl) piperidine-2-carboxylic acid (PPCA) by the method of Nakamura and Suyama (25). The HPLC system consisted of a SHIMADZU (Kyoto, Japan) LC-6A pump, a SPD-6AV UV-VIS spectrophotometric detector, a C-R5A data station. Separation was performed with a LiChrosper 100 RP-18 125-4 reverse-phase column (Merck, Darmstadt, Germany). A guard column (LiChrosper 100 RP-18, 4×4 mm I.D., 5µm particle size; Merck, Darmstadt, Germany) was placed just before the inlet of the analytical column to reduce contamination of the analytical column. The mobile phase was composed of 0.1 M phosphate buffer-acetonitrile (5:1, v/v) containing 20 mM SDS (final pH 3.95). The flow-rate was 1.0 ml/min. The UV signal was monitored at 260 nm which corresponds to an absorption maximum for PPCA.

**Mass Spectrometry**

Positive ion fast atom bombardment (FAB)-mass spectra were obtained using a JEOL JMS700 mass
spectrometer operated at an accelerating voltage of +10 kV. The FAB gun was operated at an accelerating voltage of 6 keV with an emission current of 10 mA using xenon atoms as the bombarding gas. The sample was mixed with glycerol/water.

**UV Spectrometry**

The UV spectra for DP and IDP were obtained with a UV-2100S spectrophotometer (Shimadzu, Kyoto, Japan).

**NMR Spectroscopy**

Structural assignments were made from sample solutions in D$_2$O. NMR measurements were performed at 20°C using a Varian Inova 600 MHz spectrometer operating at 600 and 150 MHz for $^1$H and $^{13}$C NMR experiments, respectively. $^1$H and $^{13}$C chemical shifts were internally referenced to dioxane at 3.5 ppm and 66.3 ppm, respectively. The water resonance was attenuated by pre-saturation during the 0.5-s relaxation delay. $^1$H resonance assignments were obtained using $^1$H-$^1$H correlated spectroscopy ($^1$H-$^1$H COSY). Data matrix was 512 real by 2048 complex for the $^1$H-$^1$H COSY experiment. Simultaneous $^1$H and $^2$H decoupling of $^{13}$C was achieved with a WALTZ-16 pulse sequence during relaxation ($^1$H) and acquisition ($^1$H, $^2$H). The $^{13}$C resonance from carbon with a directly attached proton was assigned using heteronuclear single quantum coherence (HSQC) spectroscopy. HSQC spectroscopy was the pulse field gradient standard Varian program GHSQC. The $^{13}$C resonances from carbons without a directly attached proton were assigned using heteronuclear multiple-bond coherence (HMBC) spectroscopy. HMBC spectroscopy was the pulse field gradient standard Varian program GHMQC. Carbon multiplicity was established by distortionless enhancement by polarization transfer (DEPT).
RESULTS

Isolation of DESP and IDP — DESP and IDP were isolated from acid hydrolysates of bovine ligamentum nuchae elastin by normal- and reversed-phase chromatography. DESP and IDP were well separated by reverse-phase (ODS) column chromatography using a solvent of water containing 0.02 N HCl, which the general solvent system for peptides, suggesting that those compounds are polyfunctional amino acids. The purities of DESP and IDP were confirmed by TLC and analytical HPLC analyses. DESP and IDP were completely separated by TLC as heavy ninhydrin single spots ($R_f$ of both compounds was 0.08; $R_f$ of both DES and IDE was 0.0). With HPLC elution systems that will resolve IDE and DES, DESP and IDP were ($R_t$ = 20.2 and 19.0 min, respectively) eluted before DES ($R_t$ = 34.7 min) and IDE ($R_t$ = 24.7 min) and fractionated as single peaks (FIG. 1). DESP and IDP were hygroscopic, white solids with a faint yellow tinge, and were soluble in aqueous solvents but not in dry methanol.

Structural Elucidation of DESP and IDP — The UV spectra of DESP and IDP are shown in FIG. 2. DESP exhibited absorption maximum at 264.8 nm in 0.1 N HCl. The UV spectrum was reversibly shifted in 0.1 N NaOH. The UV absorption spectrum of DESP was characteristic of 3,4,5- trisubstituted pyridine, such as, 4-ethyl-3, 5-dimethylpyridine (28). IDP exhibited absorption maximum at 272.0 nm in 0.1 N HCl. The absorption maximum was reversibly shifted in 0.1 N NaOH to 271.1 nm. The absorption spectrum of IDP was characteristic of 2,3,5- trisubstituted pyridine, such as, 2-ethyl-3, 5-dimethylpyridine (28). Crucial structural information was obtained from the $^1$H NMR (in D$_2$O). Peaks in the aromatic region are sharp singlets; comparison with DES and IDE indicated that DESP and IDP are 3,4,5- and 2,3,5- trisubstituted pyridines, respectively. A schematic drawing of DESP and IDP is shown in FIG.3, respectively, and indicates the numbering system used. The proton assignments were completed using $^1$H-$^1$H COSY experiments, shown in FIG.3B and 3D. The $^1$H-NMR spectrum of DESP showed the following signals; $\delta$H (D$_2$O): 1.624 (12, 2H), 1.818 (13, 2H), 2.090 (8, 2H), 2.090 (17, 2H), 2.818-2.927 (7, 2H), 2.818-2.916 (16, 2H), 2.807-2.916 (11, 2H), 3.810 (9, 1H, t), 3.810 (14, 1H, t), 3.908 (18, 1H), 8.374 (2, 1H, s), 8.374 (6, 1H, s). $^1$H-NMR (in D$_2$O) for DESP is shown in Fig. 3A. The triplet at 3.908 ppm and the proton signals at 3.810 ppm suggested the presence of three $\alpha$-protons compatible with the presence of three amino acids. As shown in FIG. 3B, these $\alpha$–protons showed a spin-spin interaction between aliphatic...
protons at 2.090 and 1.818 ppm, respectively. The protons signals at 2.818-2.927, 2.818-2.916 ppm suggested the presence of methyl groups on the pyridine ring. These methyl proton signals showed a spin-spin interaction between aliphatic protons at 2.090 and 1.624 ppm, respectively. The proton singlet at 8.374 ppm suggested the presence of two aromatic protons in the pyridine molecule with substitutions in symmetric positions 2 and 6, by comparing the ratio of the areas under ¹H NMR. The ¹H-NMR spectrum of IDP showed the following signals; δH (D₂O): 1.592-1.605 (8, 2H), 1.823 (9, 2H), 2.018 (17, 2H), 2.030 (13, 2H), 2.705 (12, 2H), 2.722 (16, 2H), 2.892 (7, 2H, t), 3.744 (10, 1H), 3.758 (14, 1H), 3.817 (18, 1H, t), 8.144 (4, 1H, s), 8.248 (6, 1H, s). ¹H NMR (in D₂O) for IDP is shown in FIG.3C. The three coupled triplets at 3.744, 3.758, and 3.817 ppm suggested the presence of three α-protons compatible with the presence of three amino acids. These three α-protons showed a spin-spin interaction between aliphatic protons at 1.823, 2.030, and 2.018 ppm. The triplet at 2.892 ppm and the proton signals at 2.705, 2.722 ppm suggested the presence of three methyl groups on the pyridine ring. These methyl protons showed a spin-spin interaction between aliphatic protons at 1.592 and 1.605, 2.030, and 2.018 ppm, respectively, as revealed by a ¹H-¹H COSY experiment. The two proton singlets in the aromatic region (δ 8.144 and δ 8.248) suggested the presence of two aromatic protons in the pyridine molecule with substitutions in asymmetric positions 4 and 6. The ¹³C-NMR resonances of DESP and IDP were assigned using a combination of GHSQC and GHMBC spectra. The ¹³C-NMR spectra of DESP and IDP are shown in Fig.4A and 5A, respectively. All the resonances of proton-attached carbons were readily assigned based on the one-bond ¹H-¹³C correlation cross-peaks, in the GHSQC spectrum shown in FIG.4B and 5B. The assignments were confirmed by two-bond and three-bond ¹H-¹³C correlation cross-peaks in the GHMBC spectra, as shown in FIG.4C and 5C. The ¹³C resonance of the nonproton-attached carbons was also assigned based on the cross-peaks, in the GHMBC spectra. The ¹³C-NMR spectra of DESP showed the following signals; δC (D₂O): 25.853 (C-12), 26.350 (C-7, C-16), 30.332 (C-13), 31.349 (C-8, C-17), 47.462 (C-11), 54.225 (C-9, C-18), 54.393 (C-14), 139.577 (C-2, C-6), 139.789 (C-3, C-5), 160.836 (C-4), 173.538 (C-10), 173.830 (C-15, C-19). In DESP, all of the carbons on the aromatic ring showed resonance in the chemical shift range of 139.577 to 160.836 ppm. The ¹³C resonances at 139.577 ppm had GHSQC cross-peaks to the H2 and H6 protons. The only GHMBC cross-peak from ¹³C resonances at 139.577 ppm was to the H7 proton resonance. This demonstrated that the resonance at 139.577 ppm was from the C2 carbon. The only GHMBC cross-peak from ¹³C resonances at 139.577 ppm was to the H16 proton resonance. This demonstrated that the resonance at 139.577 ppm was from the C6 carbon. The
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\(^{13}\)C resonance at 139.789 ppm had no cross-peaks in the GHSQC spectrum. The resonance at 139.789 ppm was assigned to both the C3 and C5 carbons since it is the only \(^{13}\)C resonance that has GHMBC cross-peaks to both the H8 and H17. The \(^{13}\)C resonance at 160.836 ppm was assigned to the C4 carbon since it is the only \(^{13}\)C resonance that has GHMBC cross-peaks to the following protons: H2, H6, H7, and H16. The \(^{13}\)C-NMR spectra of IDP showed the following signals: \(\delta C (D_2O): 25.054 \text{ (C-8)}, 27.045 \text{ (C-16)}, 28.009 \text{ (C-12)}, 30.363 \text{ (C-9)}, 30.537 \text{ (C-7)}, 31.152 \text{ (C-17)}, 31.363 \text{ (C-13)}, 53.879 \text{ (C-10)}, 54.104 \text{ (C-14, C-18)}, 139.007 \text{ (C-5)}, 139.584 \text{ (C-3)}, 139.420 \text{ (C-6)}, 148.025 \text{ (C-4)}, 153.058 \text{ (C-2)}, 173.415 \text{ (C-19)}, 173.714 \text{ (C-15)}, 173.512 \text{ (C-11)}\). In IDP, all of the carbons on the aromatic ring showed resonance in the chemical shift range of 139.007 to 153.058 ppm. The resonances of the C4 and the C6 carbons were easily assigned based on the GHSQC. That is, the resonances at 148.025 ppm and 139.420 ppm were assigned to the C4 and C6 carbon, respectively. The resonance at 153.058 ppm was assigned to the C2 carbon since it is the only \(^{13}\)C resonance that has GHMBC cross-peaks to both the H4 and H6 protons on the aromatic ring. The resonance at 139.007 ppm was assigned to the C5 carbon since it is the only \(^{13}\)C resonance that has GHMBC cross-peaks to both the H6 and H17 protons. The resonance at 139.584 ppm was assigned to the C3 carbon since it is the only \(^{13}\)C resonance that has GHMBC cross-peaks to both the H4 and H13 protons. The FAB-mass spectrum on glycerol matrix showed a molecular ion at \(m/z\) of 397 (M+H), as shown in FIG. 6. High resolution FAB-MS in the positive ion mode showed the molecular mass of 397.2. This corresponded to the molecular formula \(C_{18}H_{28}N_4O_6\) and was in agreement with the proposed structure. Based on these characteristics, DESP and IDP were determined as 3,4,5- and 2,3,5- trisubstituted pyridine cross-links, respectively.

**Formation of DESP and IDP from the Chichibabine reaction between allysine and ammonia** — The cross-linking in elastin involves allysine (the aldehyde produced by oxidative deamination of lysine residues) as well as intact lysine residues. A mechanism featuring the formation of DES and IDE has been proposed by some investigators (29-32). In the present study, two novel cross-linking amino acids, DESP and IDP, have three amino acid groups and pyridine skeletons. DESP and IDP, were determined to be 3,4,5- and 2,3,5- trisubstituted pyridine, respectively, by structural analyses. These structures were similar to those of DES and IDE, respectively, as shown in FIG.7. The studies with model systems have shown the detail cross-linking mechanism of DES and IDE (29, 30). DES can be formed from the condensation of the \(\alpha,\beta\)-unsaturated imine,
dehydromerodesmosine and the aliphatic aldimine, dehydrolysiononorleucine (29, 30). IDE can be formed from the condensation of 2 moles of allysine and the aliphatic aldimine, dehydrolysiononorleucine (29, 30). Judging from the mechanisms for the formation of DES and IDE, we hypothesized that the formation of DESP and IDP involved ammonia, allysine and allysine aldol.

To determine whether DESP and IDP are formed during the reaction of ammonia with the elastin, the elastin powder (50 mg) was incubated with 1M NH₄Cl in 5 ml of 0.2 M phosphate buffer under physiological conditions (pH 7.4, 37°C). Following acid hydrolysis (6 N HCl; 48h at 110°C), the formation of DESP and IDP was detected by HPLC analysis, based on the co-elution with the standard of DESP and IDP, respectively. As shown in Fig. 8A, a time-dependent increase in the amount of DESP and IDP was observed. After 28 days of incubation, 0.44 and 0.17 nmol/mg protein of DESP and IDP, were formed. After 28 days of incubation, 1.87 nmol/mg of allysine was decreased (Fig. 8B). In this experiments, the decrease of allysine-aldol was not detected after 28 days of incubation, by the HPLC analysis.

In vitro experiments at 60°C were also carried out. As shown in Fig. 8C, DESP and IDP were formed linearly with the incubation; the formation of 0.80 nmol/mg DESP and 1.66 nmol/mg IDP was observed after 14 days of incubation. A time-dependent decrease of allysine was observed; the allysine content decreased to about 76% of the control level, after 14 days of incubation (Fig. 8D). In this experiments, the decrease of allysine-aldol was not detected after 14 days of incubation, by the HPLC analysis.

Formation of DESP and IDP in vivo — From in vitro experiments, two pyridine cross-links (DESP, IDP) may be formed from the Chichibabine reaction between 3 moles of allysine and 1 mol of ammonia. The main source of ammonia for the formations of pyridine cross-links in vivo, however, has remained obscure. In vivo, deamination of α-amino group of amino acids produces ammonia, a toxic metabolite that is detoxified by conversion into urea via the urea cycle in the liver. In general, the concentration of ammonia is the highest in the portal vein. Under chronic liver failure, liver cirrhosis, the blood flow of portal vein is drained in the systemic circulation after portal-systemic shunting; it is well-known that the blood ammonia levels are elevated in liver cirrhotic patients. CCl₄-induced cirrhotic rats represents a valuable model for studying the causes and possible therapeutic prevention of hyperammonemia (33, 34). In our study, we have used rats with CCl₄-induced liver cirrhosis, to further investigate the potential mechanism for the formations of pyridine cross-links in vivo.
As shown in TABLE 1, the IDP content in the rat aorta elastin after long-term CCl₄ treatment (4 weeks), was significantly higher ($p < 0.05$) than that observed in the control rat. Also, the allysine content decreased as compared with that in the control rat (TABLE 1). No DESP could be detected in both aorta elastin of control and cirrhotic rats, by use of the RP-HPLC analytical procedure described in FIG. 1.

Detection of DESP and IDP in vivo — Bovine tissue elastin was analyzed for their DESP and IDP content using the RP-HPLC analytical procedure described in FIG. 1. The peak identities were confirmed by co-elution with the standard. Contents of DESP, IDP, allysine, DES, IDE, and allysine-aldol in bovine tissues from different organs were shown in TABLE 2. DESP and IDP were found to be distributed in all of bovine tissues containing DES and IDE. To assess the biological meaning of pyridine cross-links, we investigated the ratio of DESP:DES and IDP:IDE in bovine tissue elastin. Both ratios exhibited a high value in aorta elastin. We investigated the age-related changes of human aorta elastin cross-links, such as DESP, IDP, DES, and IDE, and of possible precursors of pyridine cross-links, allysine and allysine-aldol (FIG. 9). The DES, IDE, allysine and allysine-aldol contents of aorta elastin decreased gradually with age. The DES and IDE contents were decreased gradually with age.
DISCUSSION

The discovery of pyridine cross-links in bovine ligamentum nuchae elastin is the first molecular evidence for the involvement of ammonia and allysine in elastin cross-linking. We have named these pyridine cross-links, desmopyridine (DESP) and isodesmopyridine (IDP), respectively, since these pyridine cross-links were 3,4,5- and 2,3,5- trisubstituted pyridine and were similar to quaternary pyridinium skeletons of desmosine (DES) and isodesmosine (IDE).

The condensation of aldehydes, ketones, α,β-unsaturated carbonyl compounds or various compounds with ammonia or its derivatives to form substituted pyridines is known as Chichibabine reaction (22, 23). In vitro experiments under physiological conditions, showed that allysine was decreased with an increase in the amount of DESP and IDP in elastin, after 28 days of incubation with 1M NH₄Cl. If DESP and IDP are formed from the Chichibabine reaction between 3 moles of allysine and 1 mole of ammonia, the decrease of allysine is estimated as 1.83 nmol/mg from the formation of DESP (0.17 nmol/mg) and IDP (0.44 nmol/mg): \( \frac{0.17}{3} + \frac{0.44}{3} = 1.83 \). This estimated value is close to the experimental value (1.87 nmol/mg). After 14 days of incubation at 60°C, the ratio of formed IDP to DESP was 2.0. In the Chichibabine reaction with phenylacetaldehyde, the ratio of 2,3,5- to 3,4,5- trisubstituted pyridine formed was 2.0 (23). This value is close to that of IDP/DESP (0.44/0.17) formed after 28 days of incubation at 37°C, that of IDP/DESP (1.66/0.80) formed after 14 days of incubation at 60°C, and is also close to that of IDP/DESP (0.50/0.29) in elastin from bovine ligamentum nuchae. Two pyridine cross-links (DESP, IDP) may be formed from the Chichibabine reaction between 3 moles of allysine and 1 mole of ammonia. The over-all reaction is shown in FIG. 10.

1, 4-Dihydrodesmosine and 1, 2-dihydroisodesmosine are dihydropyridines which are believed to be the immediate biosynthetic precursors of DES and IDE, respectively (30). 1, 2-Dihydroisodesmosine can, in theory, arise via two different pathways: by addition of allysine to dehydrolysinonorleucine; or by Michael addition of lysine to the allysine-aldol. Similarly, 1, 2-dihydrodesmosine can, in theory, arise by a pathway involving aldol addition of dehydrolysinonorleucine to allysine or by a pathway involving the α,β-unsaturated imine, dehydromerodesmosine formation between lysine and the allysine-aldol. Akagawa and Suyama (29) have proposed that allysine-aldol is a possible precursor of DES and IDE, by the studies with the model system. Thus allysine–aldol can be a possible precursor of DESP and IDP. However, the involvement of allysine-aldol in the
formation of DESP and IDP couldn’t be shown in this study.

By sequence studies of elastin, Mecham and Foster (35) have shown that some or perhaps all allysine residues are not restricted to the alanine-enriched sites for other elastin cross-links, and exist in the sequence, -Gly-Ala-Glu-Allysine-(Glu)-, creating regions of negative charge from glutamic acid residues. Since ammonia exist as an ammonium ion of positive charge in solution, it seems to access easily to the sites containing allysine residue. In elastin, the amount of allysine residues are also much higher than that of allysine-aldol, as seen in TABLE 2. Thus the formation of DESP and IDP may be associated with the Chichibabine reaction between 3 moles of allysine and ammonia. In this study, however, the involvement of allysine-aldol in these formation can’t be denied as described above.

It is known that pyridine compounds are formed by the Hoffmann degradation of quaternary pyridinium compounds under high temperature (36, 37). No both DESP and IDP were found to be formed from DES and IDE, respectively, by prolong acid hydrolysis under the condition of 110°C for 240 h in 6N HCl (results not shown). DESP and IDP, therefore, aren’t artifacts formed from DES and IDE, respectively, by acid hydrolysis. This may also suggest that assembled DES and IDE in elastin are very stable and aren’t subject to the Hoffmann degradation.

To obtain more information on the formation of pyridine cross-links, in vivo experiment was performed using CCL₄-induced liver cirrhotic rats. This animal model seems to be well suited for hyperammonemia in patients with liver cirrhosis. Hyperammonemia causes dysfunction of multiple organs in patients with cirrhosis. We have investigated the changes of IDP and allysine content in the aorta elastin. IDP was found to be formed in the rat aorta elastin, by long-term CCl₄ treatment (4 weeks), consistent with the decrease of allysine residue (TABLE 1). IDP may be formed by the Chichibabine reaction both allysine residues in elastin and ammonia elevated in blood associated with chronic liver failure; IDP may be an abnormal cross-link in elastin.

Kidney failure frequently accompanies liver failure. Although in cirrhotic patients the kidney continued to release ammonia into the circulation (38, 39), this release decreased at elevated ammonia concentrations (38). Similarly, artificial hyperammonemia in healthy volunteers turned the kidney into an organ of net ammonia uptake from the circulation (40), also suggesting enhanced renal ammonia excretion. Urine production is markedly diminished as cirrhosis worsens. In end-stage cirrhosis, complete kidney shut down (hepato-renal syndrome) is usually fatal. Therefore, under this condition, DESP and IDP contents may increase in the aorta.
elastin and the aorta elastin may be damaged by ammonia.

Connective tissues in the aorta are known to be greatly affected by age because of impaired functional properties and increased susceptibility to diseases. Elastin and collagen are major important extracellular matrix proteins in providing the aorta with tensile strength and elasticity. The ratio between elastin and collagen play key roles in conditioning the morphological-functional properties of the aorta. During aging and senescence, the aorta becomes stiffer and its elasticity is reduced. The age-related changes in stiffness and elasticity have been ascribed to these changes in collagen and elastin concentrations in the aorta. In general, the elastin concentration decreases, and the collagen concentration increases until a certain age and then reaches a plateau (41-45). However, few studies have investigated the biochemical properties and the contents of elastin and collagen in the same vessel, and the results have been conflicting (41, 42, 45). The changes in the biochemical properties of collagen are characterized by the formation of cross-links, especially those derived from non-enzymatic glycation (19, 20). Unlike collagen, elastin is formed only in developing tissue, with little or no synthesis in adults (46). Thus, human aorta elastin shifts from an anabolic state to catabolic state with aging (47). All elastin cross-links are gradually reduced with age (47). The age-related formation of cross-links in aorta elastin has not been reported. We found that the pyridine cross-links reported in the present paper, was increased with age (FIG. 9). Thus, these distribution and gradual increased levels may indicate the functional changes of the aorta elastin damaged by ammonia with aging and senescence.

Studies on the distribution of DESP and IDP and on the DESP/DES and IDP/IDE ratios in the aorta elastin under various conditions may provide new insights into the chemical changes of elastin in aorta. We have been investigating the biological meaning of the presence of higher pyridine cross-links in the aorta, than in other tissue elastin. In normal cross-linking of elastin, it is well known that 1 mole of ammonia is produced when the fomation of allysine residues from lysine residues by lysyl oxidase. Thus additional studies containing the participation of lysyl oxidase, will be also needed to determine the source of ammonia, which is involved in the formation of pyridine cross-links (DESP, IDP).
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REFERENCES


FOOTNOTES

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1Abbreviations used: APC, allysine-bis-p-cresol derivative; DEPT, distortionless enhancement by polarization transfer; DESP, desmopyridine; DES, desmosine; GHMBC, pulse field gradient heteronuclear multiple-bond coherence spectroscopy; GHSQC, pulse field gradient heteronuclear single quantum coherence spectroscopy; 'H-1H COSY, 'H-2H correlated spectroscopy; IDP, isodesmopyridine; IDE, Isodesmosine.
FIGURE LEGENDS

FIG. 1. Representative HPLC profiles of bovine ligamentum nuchae elastin (A), DESP (B) and IDP standards (C). Bovine ligamentum nuchae elastin was hydrolyzed in 6 N HCl. The analytical HPLC condition was performed with a reverse-phase column (Mightysil RP-18 150×4.6) using a solvent of 0.1 M sodium phosphate buffer/acetonitrile (5:1) containing 20 mM SDS, at pH 3.6.

FIG. 2. UV absorption spectra of DESP (upper) and IDP (lower). UV absorption spectra were monitored in 0.1 N HCl, 0.1 N NaOH and 0.1 N HCl in this order.

FIG. 3. $^1$H- (A), $^1$H-$^1$H COSY (B) NMR spectra of DESP and $^1$H- (C) and $^1$H-$^1$H COSY (D) NMR spectra of IDP. NMR measurements were performed at 20°C using a Varian Inova 600 MHz spectrometer. $^1$H chemical shift was internally referenced to dioxane at 3.5 ppm. The water resonance was attenuated by pre-saturation during the 0.5-s relaxation delay. The inset (A) and (C) show the structures of DESP and IDP, respectively.

FIG. 4. $^{13}$C- (A), $^1$H-$^{13}$C HSQC (B) and $^1$H-$^{13}$C HMBC (C) NMR spectra of DESP. NMR measurements were performed at 20°C using a Varian Inova 600 MHz spectrometer operating at 600 and 150 MHz for $^1$H and $^{13}$C NMR experiments, respectively. $^1$H and $^{13}$C chemical shift were internally referenced to dioxane at 3.5 ppm and 66.3 ppm, respectively. The water resonance was attenuated by pre-saturation during the 0.5-s relaxation delay. $^1$H-$^{13}$C HSQC spectroscopy was the pulse field gradient standard Varian program GHSQC. $^1$H-$^{13}$C HMBC spectroscopy was the pulse field gradient standard Varian program GHMQC.

FIG. 5. $^{13}$C-(A), $^1$H-$^{13}$C HSQC (B) and $^1$H-$^{13}$C HMBC (C) NMR spectra of IDP. NMR measurements were performed at 20°C using a Varian Inova 600 MHz spectrometer operating at 600 and 150 MHz for $^1$H and $^{13}$C NMR experiments, respectively. $^1$H and $^{13}$C chemical shift were internally referenced to dioxane at 3.5 ppm and 66.3 ppm, respectively. The water resonance was attenuated by pre-saturation during the 0.5-s relaxation delay. $^1$H-$^{13}$C HSQC spectroscopy was the pulse field gradient standard Varian program GHSQC. $^1$H-$^{13}$C HMBC spectroscopy was the pulse field gradient standard Varian program GHMQC.
FIG. 6. **FAB mass spectra of DESP (upper) and IDP (lower).** DESP and IDP were purified and analyzed by positive FAB-MS. The signal patterns of both compounds were extremely similar and FAB-high resolution mass spectra gave the molecular formula $C_{18}H_{28}N_4O_6$ for each compounds.

FIG. 7. **Comparisons of the structures of normal cross-links (DES, IDE) and abnormal cross-links (DESP, IDP, OXD, and IOXD) found in elastin.** OXD and IOXD were isolated from the acid hydrolysates of the bovine aortic elastin, and identified to have N-substituted 1, 2-dihydro-2-oxypyridine and N-substituted 1, 4-dihydro-4-oxopyridine skeletons, respectively, with three $\alpha$-amino acid groups and mass of 495 ($C_{23}H_{37}N_5O_7$) (20, 21). Recently, we found that OXD and IOXD are oxidative metabolic intermediates generated from DES and IDE *in vitro*, respectively, by reactive oxygen species (21).

FIG. 8. **Changes of DESP, IDP, and allysine in bovine ligamentum nuchae elastin (10 mg/ml) incubated with 1 M NH$_4$Cl under physiological conditions (A, B, pH 7.4, 37°C), and at 60°C (C, D, pH 7.4).** DESP (open circles), IDP (solid circles), and allysine (solid triangles) were assayed as described under “EXPERIMENTAL PROCEDURES”. The control experiments without NH$_4$Cl showed no changes of DESP, IDP, and allysine contents under both conditions (37°C and 60°C). Other experimental details are described in “EXPERIMENTAL PROCEDURES”. Values shown are means ± S.D. of three independent determinations.

FIG. 9. **Cross-links and cross-link precursors contents in human aorta elastin as a function of age.** The inset (A) shows the enlarged view of age-related changes of DESP and IDP. Measurements of DES, IDE, DESP and IDP were conducted as described in the legends of FIG. 1 and other experimental details are described in “EXPERIMENTAL PROCEDURES”. The assays for allysine and allysine-aldol were conducted as described in “EXPERIMENTAL PROCEDURES”.

FIG. 10. **The over-all reaction for the formation of DESP (upper) and IDP (lower).**

R, side chain of amino acid.
**TABLE 1. Content of IDP and allysine in the aorta elastin from control and liver cirrhotic rat.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Liver cirrhosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDP</td>
<td>5.9±1.5</td>
<td>11.1±0.9</td>
</tr>
<tr>
<td>Allysine</td>
<td>7.8±0.7</td>
<td>5.8±0.5</td>
</tr>
</tbody>
</table>

All data are expressed as nmol per mg elastin and represents the mean ±S.D. (n=3). IDP was analyzed according to the legends of Fig. 1. Assay for allysine was conducted according to "EXPERIMENTAL PROCEDURES".

*Liver cirrhosis was induced in rats by repeated intraperitoneal injections of carbon tetrachloride/mineral oil (1:1) at a dose of 1 ml/kg of body weight three times a week (on Monday, Wednesday, and Friday) for a total of 10 injections (24).
TABLE 2
Cross-Links and cross-link precursors in elastin from different bovine tissues

<table>
<thead>
<tr>
<th></th>
<th>DESP(^a)</th>
<th>IDP(^a)</th>
<th>Allysine(^b)</th>
<th>Allysine-aldol(^c)</th>
<th>DES(^a)</th>
<th>IDE(^a)</th>
<th>DESP/DES (%)</th>
<th>IDP/IDE(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligamentum nuchae</td>
<td>0.29</td>
<td>0.59</td>
<td>47.4</td>
<td>11.0</td>
<td>14.2</td>
<td>12.1</td>
<td>2.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Aorta</td>
<td>0.55</td>
<td>1.02</td>
<td>32.0</td>
<td>3.21</td>
<td>12.9</td>
<td>11.0</td>
<td>4.5</td>
<td>9.3</td>
</tr>
<tr>
<td>Lung</td>
<td>0.11</td>
<td>0.41</td>
<td>10.7</td>
<td>1.17</td>
<td>6.57</td>
<td>6.03</td>
<td>1.7</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Note. Values are expressed as nmol per mg of elastin. The value in the table represent the average of triplicate assays for three samples.

\(^a\) Assays for DES, IDE, DESP, and IDP were conducted according to the condition of FIG. 1.

\(^b\) Assay for allysine was conducted according to conditions described in "EXPERIMENTAL PROCEDURES"(27).

\(^c\) Assay for allysine-aldol was conducted according to conditions described in "EXPERIMENTAL PROCEDURES"(25).
FIG. 10

DESOMPYRIDINE

ISODESOMPYRIDINE
Two new elastin cross-links having pyridine skeleton: Implication of ammonia in elastin cross-linking in vivo

Hideyuki Umeda, Masamichi Takeuchi and Kyozo Suyama

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