

# Imidazolone, a novel advanced glycation end product, is present at high levels in kidneys of rats with streptozotocin-induced diabetes

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**Abstract** We produced a monoclonal antibody to imidazolones A and B, novel advanced glycation end products formed from the reaction of 3-deoxyglucosone (3-DG) with the guanidino group of arginine. Liquid chromatography/mass spectrometry demonstrated that the formation of imidazolone A by incubating 3-DG with arginine is very rapid, reaching a maximum concentration within 24 h, but the formation of imidazolone B is very slow and low in quantity even after 2 weeks. Thus, at physiological conditions the formation of imidazolone A is dominant, while that of imidazolone B is negligible. Immunochemistry demonstrated that the imidazolone content in the kidneys of streptozotocin-induced diabetic rats was significantly higher than in the control rats. Serum levels of 3-DG in the diabetic rats were also significantly higher than in control rats. 3-DG attacks the arginine residues of the tissue proteins, producing imidazolone at high levels in the kidneys affected by diabetic nephropathy.

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**Key words:** Imidazolone; Advanced glycation end product; Diabetes; 3-Deoxyglucosone; Streptozotocin

## 1. Introduction

Glucose reacts non-enzymatically with protein amino groups to initiate glycation, the early stage of the Maillard reaction. This process begins with the conversion of reversible Schiff base adducts to stable, covalently bound Amadori rearrangement products. The levels of the Amadori products on numerous proteins are elevated in proportion to the degree of hyperglycemia in diabetes mellitus. In the intermediate stage of the Maillard reaction, the Amadori products can then undergo multiple dehydration and rearrangements to produce highly reactive carbonyl compounds such as 3-deoxyglucosone (3-deoxy-D-erythro-hexos-2-ulose, 3-DG) [1–4], which reacts again with free amino groups, leading to crosslinking and browning of the proteins via the formation of advanced glycation end products (AGEs) in the late stage of the Maillard reaction. AGEs are characterized by (1) browning, (2) fluo-

rescence, (3) crosslinking and (4) biological recognition through receptors specific for AGEs on macrophages [5,6]. Several compounds such as 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) [7], N<sup>ε</sup>-(carboxymethyl)-L-lysine (CML) [8], pyralline [9], pentosidine [10] and crosslines [11] have been proposed as candidates for the structures of AGEs. However, FFI [12] was reported not to be AGE but to be an artifact.

Recently, imidazolone compounds have been isolated from the incubation mixture of 3-DG and an arginine derivative as novel AGEs [13,14]. It has been reported that 3-DG first attacks arginine residues in proteins and then reacts with the lysine residues under physiological conditions at 37°C and pH 7.4 [2,4]. We produced several clones of monoclonal anti-AGE antibodies, and found that one of the antibodies specifically reacted with imidazolones.

Incubation of 3-DG with proteins also leads to the formation of pyralline [15] and pentosidine [16], which have been identified in human tissue proteins such as collagen and lens proteins. 3-DG has also been suggested to be a hydrolysis product of fructose-3-phosphate which was identified in the lens of diabetic rats [17]. These data suggest that the formation of 3-DG can occur during the Maillard reaction *in vivo* and be involved in the development of diabetic complications. Recently, we developed a sensitive and specific gas chromatography/mass spectrometry using a stable-isotope dilution method for measuring 3-DG in human serum, and found that serum 3-DG levels were elevated in not only diabetic patients [18] but also uremic patients [19,20].

In this study we first measured the tissue contents of imidazolone in streptozotocin (STZ)-induced diabetic rats to examine the pathogenic role of the Maillard reaction in the diabetic rats by enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti-imidazolone antibody we had produced.

## 2. Materials and methods

### 2.1. Samples

Serum, urine and tissue samples were obtained from 13 diabetic (21 weeks old; STZ 3 months), 10 non-diabetic (9 weeks old; control 0 month) and 10 non-diabetic (21 weeks old; control 3 months) Wistar male rats. The diabetic rats were made diabetic at the age of 9 weeks by intravenous injection of 30 mg/kg STZ. Plasma glucose was measured after 1 month, and only animals exhibiting hyperglycemia (higher than 20 mM) were selected for the study. Three months after injection, the samples of serum, urine, and kidneys were obtained. Before cutting out the tissues, perfusion with phosphate buffer (0.01 M) at about 30 ml/min for 3–5 min was done by catheterization from the thoracic aorta through the hepatic vein to remove blood from the kidneys.

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**Abbreviations:** 3-DG, 3-deoxyglucosone; AGEs, advanced glycation end products; CML, N<sup>ε</sup>-(carboxymethyl)-L-lysine; STZ, streptozotocin; Fru-Lys, N<sup>α</sup>-t-Boc-N<sup>ε</sup>-fructosyl-L-lysine; Tos-Lys-Me, N<sup>α</sup>-p-tosyl-L-lysine-methyl ester; Benz-Arg-amide, N<sup>α</sup>-benzoyl-L-arginine amide; imidazolone A, 2-(4-amino-5-pentanoic acid)-amino-5-(2,3,4-trihydroxybutyl)-4(5H)-imidazolone; imidazolone B, 2-(4-amino-5-pentanoic acid)-amino-5-(2,3,4-trihydroxybutyl)-4-imidazolone; LC/MS, liquid chromatography/mass spectrometry

## 2.2. Serum 3-DG

Serum 3-DG levels were measured according to the method we have previously reported [18]. The intra- and interassay coefficients of variation were 7.8% ( $n=5$ ) and 8.6% ( $n=5$ ), respectively, at 1.79  $\mu\text{M}$ , and 1.6% ( $n=5$ ) and 5.0% ( $n=5$ ), respectively, at 12.5  $\mu\text{M}$ . Recoveries of 3-DG spiked to a serum sample at concentrations of 3.09  $\mu\text{M}$  and 12.3  $\mu\text{M}$  were  $109.9 \pm 11.5\%$  (mean  $\pm$  S.D.,  $n=5$ ) and  $87.0 \pm 1.6\%$  (mean  $\pm$  S.D.,  $n=5$ ), respectively.

## 2.3. In vitro preparation of AGE-modified proteins and lysine analog, and AGE structures

AGE-modified keyhole limpet hemocyanin (AGE-KLH), AGE-modified human serum albumin (AGE-HSA), and AGE-modified  $\beta_2\text{m}$  (AGE- $\beta_2\text{m}$ ) were produced by incubating KLH (20 g/l), HSA (100 g/l), or  $\beta_2\text{m}$  (100 mg/l) (all from Sigma Chemical Company, St. Louis, MO, USA), respectively, at 37°C for 1 month (HSA,  $\beta_2\text{m}$ ) or 3 months (KLH) with 1 M glucose in 0.2 M phosphate buffer, pH 7.4, containing 0.02%  $\text{NaN}_3$ .

An Amadori product,  $N^\alpha$ -t-Boc- $N^\epsilon$ -fructosyl-L-lysine (Fru-Lys), was produced by refluxing  $N^\alpha$ -t-Boc-L-lysine (100 mg, Kokusan Chemical Works, Tokyo, Japan) with glucose (200 mg) in methanol (10 ml) at 80°C for 2 h, followed by evaporation and subsequent purification with silica column chromatography by eluting with a solution composed of methanol/ethyl acetate/acetic acid (60:39:1, v/v).

$N^\alpha$ - $p$ -Tosyl- $N^\epsilon$ -(carboxymethyl)-L-lysine-methyl ester (Tos-CML-Me) was synthesized as reported by us [21]. CML derivative of BSA (CML-BSA) was synthesized as reported by Kato et al. [22]. Pentosidine was synthesized according to the method of Sell and Monnier [23]. Caproyl pyrroline was synthesized according to the method of Hayase et al. [24]. Tos-CML-Me, pentosidine and caproyl pyrroline were confirmed to be pure using nuclear magnetic resonance spectrometry and mass spectrometry.

3-DG (1 M) was incubated with 2 M  $N^\alpha$ - $p$ -tosyl-L-lysine-methyl ester (Tos-Lys-Me; Sigma Chemical Company, St. Louis, MO, USA) in 0.2 M phosphate buffer, pH 7.4, at 37°C for 2 months, to examine the reactivity of anti-imidazolone antibody with the incubation solution. Arginine (0.1 M) was incubated with 0.2 M glyoxal, 0.2 M methylglyoxal (all from Sigma Chemical Company, St. Louis, MO, USA) or 0.2 M 3-DG in 0.2 M phosphate buffer (pH 7.4) at 37°C for 1 month to further examine the reactivity of anti-imidazolone antibody with the incubation solutions.

## 2.4. In vitro preparation of imidazolone compounds

3-DG (0.2 M) was incubated with 0.1 M  $N^\alpha$ -benzoyl-L-arginine amide (Benz-Arg-amide; Nakarai Chemical Co., Kyoto, Japan) in 0.1 M sodium phosphate buffer, pH 7.4, at 50°C for 132 h [13,14]. Two imidazolone derivatives, 2-(4-benzoylamino-5-pentamide)-amino-5-(2,3,4-trihydroxybutyl)-4(5H)-imidazolone (a derivative of imidazolone A) and 2-(4-benzoylamino-5-pentamide)-amino-5-(2,3,4-trihydroxybutyl)-4-imidazolone (a derivative of imidazolone B) were purified from the incubation solution by high performance liquid chromatography (HPLC). HPLC was performed by monitoring at 235 nm with a Jasco UVDEC-100-VI detector (Jasco, Tokyo, Japan) with a solvent system of 20% methanol, using an ODS 4251-N column (Sensyu Kagaku, Tokyo, Japan) [13,14].

3-DG (0.2 M) was incubated with arginine (0.1 M) in 0.2 M phosphate buffer, pH 7.4, at 37°C for 2 weeks to examine the effect of incubation time on the production of imidazolone. Imidazolones A and B were formed by incubating 3-DG with arginine (see Fig. 1). Incubation solutions were obtained after incubation for 1 h, 6 h, 24 h, 1 week and 2 weeks, and analyzed by ELISA using anti-imidazolone antibody (AG-1) and liquid chromatography/mass spectrometry (LC/MS).

## 2.5. LC/MS

LC/MS was performed using a TSQ-7000 (Finnigan MAT, San Jose, CA, USA) with electrospray ionization (ESI). ESI spray voltage, capillary temperature and sheath gas ( $\text{N}_2$ ), were 4.5 kV, 252°C and 40 psi, respectively. Incubation solutions of 3-DG with arginine were introduced into the mass spectrometer by flow injection with a constant flow (0.5 ml/min) of a solution (water/methanol, 4:1, v/v). The ions characteristic for arginine ( $m/z$  175), imidazolone A ( $m/z$  319) and imidazolone B ( $m/z$  317) were monitored to determine the concentrations of the compounds in the incubation solutions. The levels of

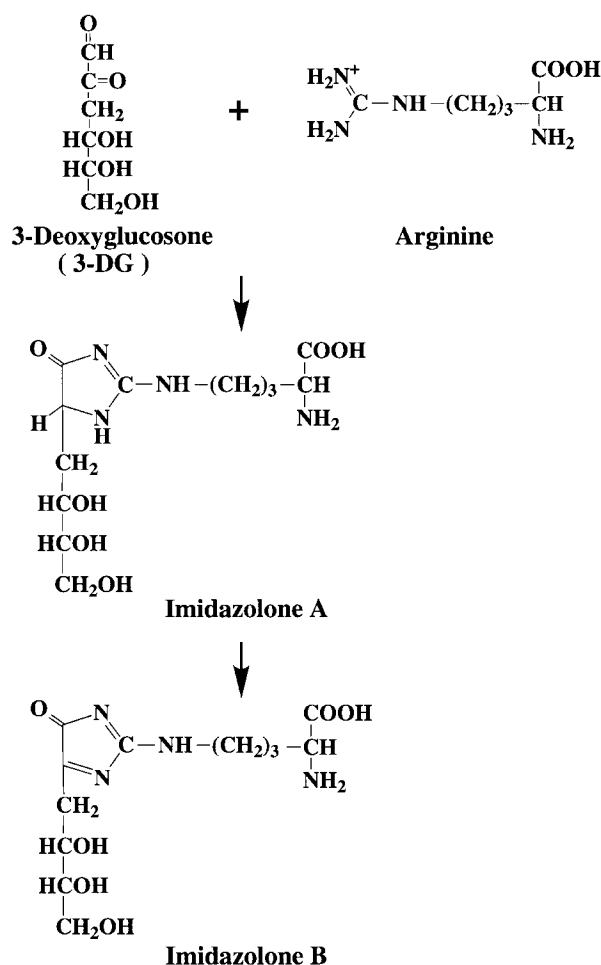


Fig. 1. Production of imidazolones A and B by incubating 3-DG with arginine. Imidazolone A was first formed, followed by its oxidation to produce imidazolone B. At physiological conditions the formation of imidazolone A is dominant, while that of imidazolone B is negligible.

arginine, imidazolone A and imidazolone B were expressed as arbitrary units in peak areas of the respective ions.

## 2.6. Production of monoclonal anti-imidazolone antibody

Monoclonal anti-AGE antibodies were produced by immunizing mice with AGE-KLH as reported in detail by us [25]. The antibodies in the culture medium were screened by sandwich ELISA using anti-mouse IgG antibody-coated plate, AGE-HSA, and peroxidase-labeled anti-HSA antibody, and then two monoclonal clone types of anti-AGE antibodies (AG-1 and AG-10) were raised. Recently, the epitope of AG-10 has been found to be CML [21].

## 2.7. Sample preparation for imidazolone measurement

Kidneys were washed with PBS and then dissected into small pieces. The tissue pieces were kept at 4°C with 1 ml of acetone/chloroform (1:1, v/v) overnight. After removing the organic solvent, the defatted tissue was incubated at 37°C for 48 h with collagenase type 7 (Sigma Chemical Company, St. Louis, MO, USA) in 20 mM Tris-HCl, pH 7.5, containing 0.1 M  $\text{CaCl}_2$  (collagenase: defatted tissue, 1:100, w/w). After centrifugation at  $10000 \times g$  for 30 min at 4°C, the supernatant was diluted five times with water and used for imidazolone measurement.

## 2.8. Competitive ELISA for imidazolone

Sample solution (25  $\mu\text{l}$ ) or standard imidazolone A (0.004–0.50 mg/l) was incubated at room temperature for 1 h in an AGE-HSA (0.01 g/

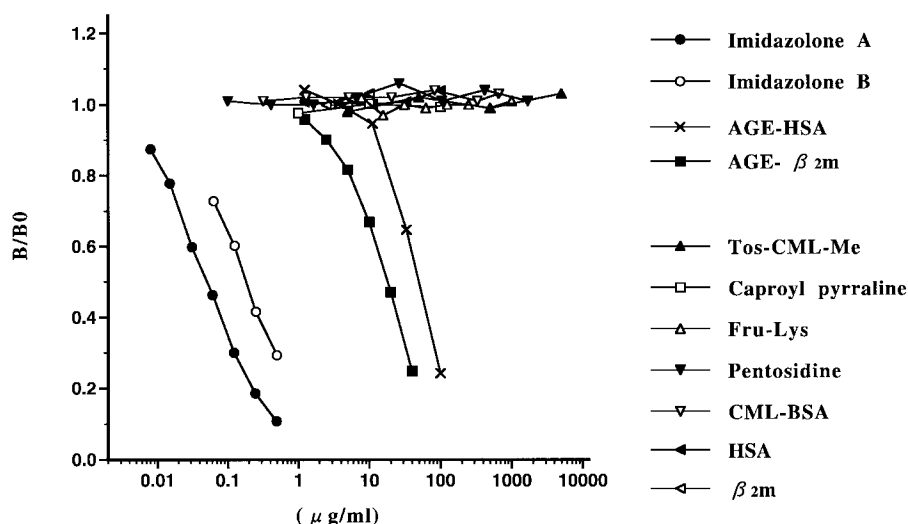


Fig. 2. Competitive ELISA demonstrating the specificity of the monoclonal anti-imidazolone antibody (AG-1). Derivative of imidazolone A: 2-(4-benzoylamino-5-pentamide)-amino-5-(2,3,4-trihydroxybutyl)-4(5*H*)-imidazolone; derivative of imidazolone B: 2-(4-benzoylamino-5-pentamide)-amino-5-(2,3,4-trihydroxybutyl)-4-imidazolone.

l)-coated microplate with PBS containing 0.25% BSA (50  $\mu$ l) and peroxidase-conjugated anti-imidazolone antibody (AG-1). After washing four times with PBS, *o*-phenylenediamine (100  $\mu$ l) was added to the microplate. Reaction was stopped by adding 1.6 M  $H_2SO_4$  (100  $\mu$ l), and absorption at 492 nm was measured using 620 nm as control. Imidazolone contents were expressed as arbitrary units (imidazolone A  $\mu$ mol per g protein measured by Lowry method). Recoveries of AGE-HSA added to the extract of kidney at concentrations of 10.9 mg/l and 21.5 mg/l were  $89.3 \pm 16.2\%$  ( $n=4$ ) and  $86.3 \pm 7.8\%$  ( $n=4$ ), respectively. Intraassay coefficients of variation for imidazolone were 12.1% ( $n=8$ ), 10.5% ( $n=8$ ), 4.8% ( $n=8$ ) and 3.6% ( $n=8$ ) for four kidney samples from one control (0 month), one control (3 months), and two STZ (3 months) rats, respectively.

#### 2.9. Measurement of the other biochemical parameters

Serum glucose was measured by an autoanalyzer using the Merck liquid GLU test kit (Kanto Kagaku, Tokyo, Japan). Serum fructosamine was measured with the fructosamine test Rosch II (Japan Rosch, Tokyo, Japan). Serum and urine creatinine were measured with a Beckman creatinine analyzer (Beckman Instruments) using the Jaffé reaction method. Creatinine clearance was measured by collecting 24 h urine. Urinary protein was measured with a micro TP-test kit (Wako Pure Chemical, Osaka, Japan) using the pyrogallol red method.

### 3. Results

#### 3.1. Characterization of monoclonal anti-imidazolone antibody

The specificity of the anti-AGE antibody (AG-1) was studied using the competitive ELISA (Fig. 2). The antibody reacted with the AGE-modified proteins such as AGE-HSA and AGE- $\beta_2m$ , demonstrating that the anti-AGE antibody recognizes a common epitope of AGE-modified proteins. The antibody did not react with Fru-Lys, an Amadori product. The antibody did not react with Tos-CML-Me, CML-BSA, caproyl pyralline or pentosidine (Fig. 2). Thus, the anti-AGE antibody was demonstrated not to react with Amadori product, or well-known AGE structures such as CML, pyralline, or pentosidine.

The epitope of the anti-AGE antibody (AG-1) was concluded to be imidazolone, since the antibody reacted with benzamide derivatives of imidazolones A and B (Fig. 2) and the incubation solutions of 3DG and Benz-Arg-amide, but did not react with 3DG or Benz-Arg-amide (Fig. 3). The antibody did not react with the incubation solution of 3-DG and Tos-

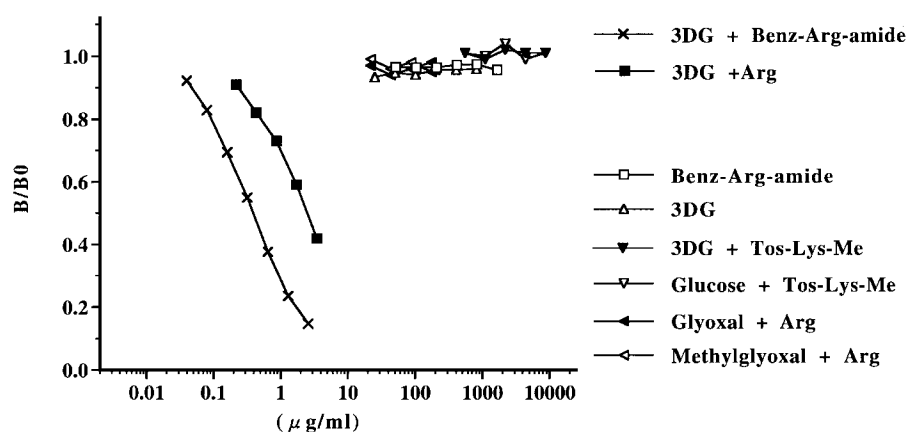


Fig. 3. Competitive ELISA demonstrating the specificity of the monoclonal anti-imidazolone antibody (AG-1). In the case of the incubation solution, the  $\mu$ g/ml of the x axis refers to the concentration of Benz-Arg-amide, Arg, or Tos-Lys-Me at the start of incubation.

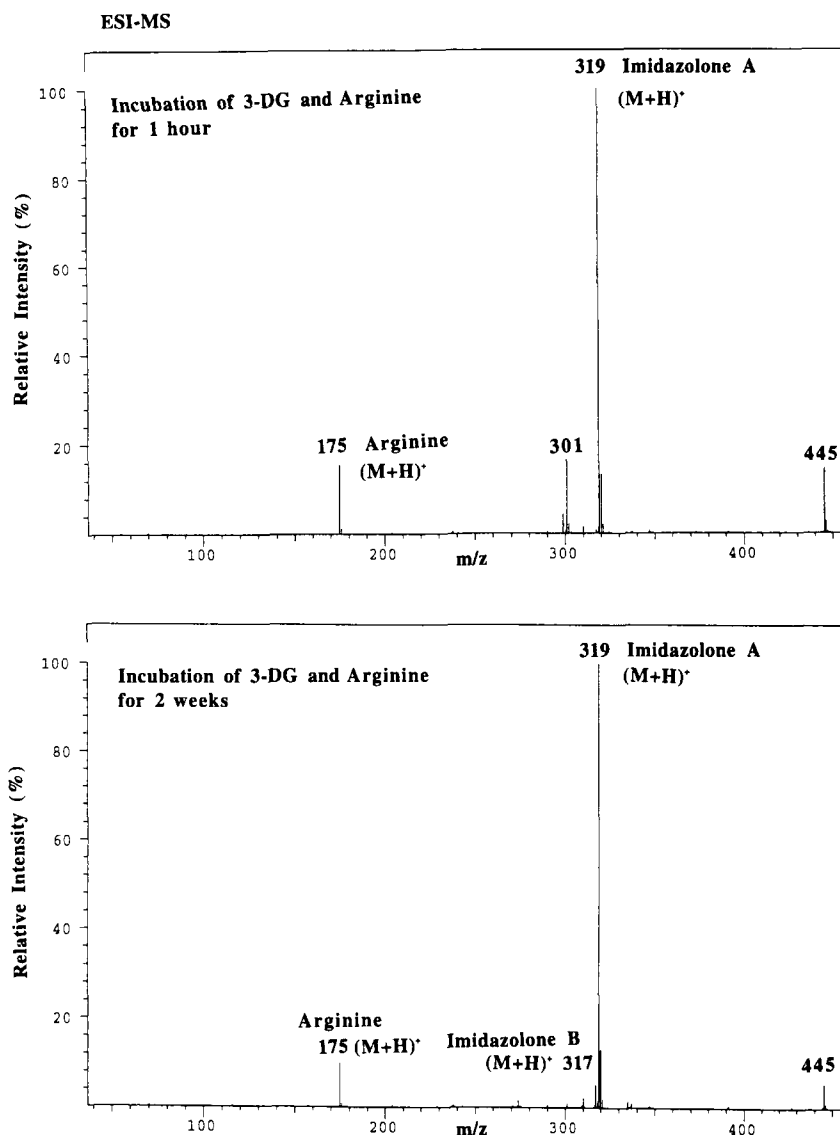


Fig. 4. ESI mass spectra of the incubation solutions of 3-DG and arginine incubated for 1 h and 2 weeks.

Lys-Me, the major product of which was CML. The antibody reacted with the incubation solution of arginine and 3-DG, but did not react with the incubation solutions of arginine and the other 1,2-dicarbonyl compounds (glyoxal or methylglyoxal) (Fig. 3). These results demonstrate that the epitope of the antibody is the imidazolone ring coupling with at least a partial structure of 3-DG, and that the direct precursor of imidazolone is 3-DG but not the other dicarbonyl compounds (glyoxal or methylglyoxal).

### 3.2. Effect of incubation time on the formation of imidazolone

Fig. 4 shows ESI mass spectra of the incubation solutions of 3-DG and arginine after incubation for 1 h and 2 weeks. The ion peak at  $m/z$  175 (M+H)<sup>+</sup> represents arginine (molecular weight 174), the ion peaks at  $m/z$  319 (M+H)<sup>+</sup> and  $m/z$  317 (M+H)<sup>+</sup> represent imidazolone A (molecular weight 318) and imidazolone B (molecular weight 316), respectively. 3-DG could not be ionized by ESI, providing no ion peak in the mass spectra. Fig. 5 shows the mass chromatograms of arginine, imidazolone A and imidazolone B in the incubation

solutions. Fig. 6 summarizes the time course of imidazolone formation as demonstrated by ELISA and LC/MS. As arginine level decreased rapidly, imidazolone level increased. A considerable amount of imidazolone A was formed even after incubation for 1 h, and its formation reached a maximum within 24 h. However, imidazolone B is formed very slowly and low in quantity, reaching a maximum level after incubation for 2 weeks. Time course of imidazolone formation estimated by ELISA was comparable to that by LC/MS, demonstrating that ELISA using the antibody is specific for imidazolone.

### 3.3. Imidazolone content in the kidneys

Table 1 shows the imidazolone content in the kidneys, and the levels of serum 3-DG and the other parameters in STZ-induced diabetic and normal rats. The diabetic rats showed nephropathy with proteinuria, hypoproteinemia, hyperlipidemia and reduced creatinine clearance. Imidazolone contents in kidneys were significantly increased in the diabetic rats as compared with control (3 months) rats.

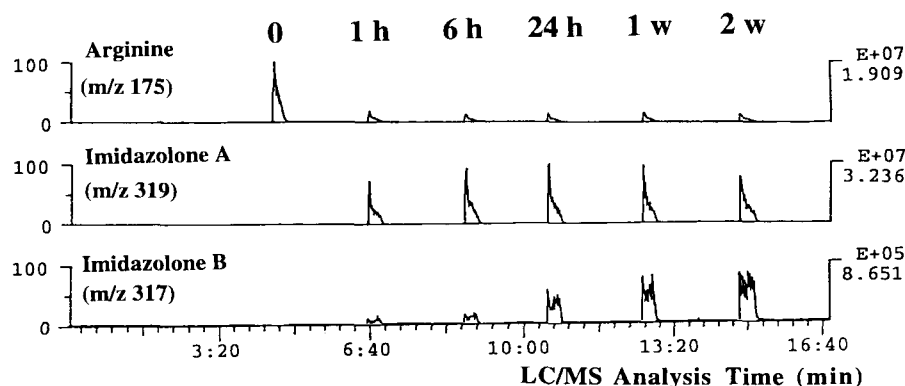


Fig. 5. Mass chromatograms of arginine, imidazolone A and imidazolone B in the incubation solutions.

#### 4. Discussion

This is the first study demonstrating that imidazolone is a common epitope of AGE-modified proteins and is present *in vivo* at high levels in the kidneys of diabetic rats with nephropathy. Since 3-DG reacts with arginine residues in proteins to induce their imidazolone modification, elevated serum levels of 3-DG result in high kidney contents of imidazolone in the STZ diabetic rats with nephropathy. The imidazolone modification of proteins in kidneys may be involved in the pathogenesis of diabetic nephropathy.

Formation of imidazolone A by incubating 3-DG with arginine is very rapid, reaching a maximum concentration within 24 h. However, the formation of imidazolone B is very slow and low in quantity (a few percent of the imidazolone A level) even after incubation for 2 weeks. This appears to be inconsistent with the report that the amount of imidazolone B formed was higher than that of imidazolone A after incubation for 6 days [14]. This discrepancy can be explained by the difference in the incubation temperature. In that report the incubation was performed at 50°C and pH 7.4 [14], while in our experiments at 37°C and pH 7.4. Our study demonstrates that at physiological conditions the formation of imidazolone A is dominant, while that of imidazolone B is negligible. Thus, imidazolone A is the dominant antigen detected by ELISA using anti-imidazolone antibody (AG-1) in the kidneys of diabetic rats.

Formation of imidazolone ring was reported in the incubation solution of an arginine residue and methylglyoxal [26]. However, our anti-imidazolone antibody does not react with

the incubation solution of arginine and methylglyoxal (or glyoxal), demonstrating that the antibody does not recognize the imidazolone ring itself. Since our antibody reacts with the incubation solution of an arginine residue and 3-DG, the antibody recognizes the imidazolone ring coupling with at least partial structure of 3-DG. The antibody is specific for 3-DG-derived imidazolone and not for methylglyoxal (or glyoxal)-derived imidazolone. Thus, the imidazolone detected using our antibody was formed by 3-DG but not by methylglyoxal nor glyoxal.

The elevated serum levels of 3-DG in the diabetic rats may be due to the increased production of 3-DG via the Maillard reaction resulting from hyperglycemia. However, serum 3-DG did not correlate with fructosamine or glucose, supporting the role of the reducing enzymes in determining the serum levels of 3-DG in the diabetic rats. 3-DG is normally detoxified by reducing enzymes to 3-deoxyfructose, which is then excreted to urine [27]. In monkey the reducing enzymes of 3-DG are composed of aldehyde reductase, aldose reductase and dihydrol dehydrogenase, and kidney showed the highest activity of the reducing enzymes of 3-DG among various tissues [28]. 3-Deoxyfructose has been detected in human urine and plasma [29] at high concentrations in diabetic patients [30]. The ability to detoxify 3-DG by the reducing enzymes may provide a genetic basis for differences in the severity of diabetic complications and age-related pathologies.

Pyrraline was detected in sclerosed glomeruli from diabetic patients by immunohistochemistry using an anti-pyrraline antibody [31]. Since pyrraline is also formed by reacting lysine residue with 3-DG, elevated serum 3-DG levels in diabetes

Table 1

Levels of tissue imidazolone, serum 3-DG and the other parameters in streptozotocin (STZ)-induced diabetic rats and control non-diabetic rats

	Control (0 month) <i>n</i> = 10	Control (3 months) <i>n</i> = 10	STZ (3 months) <i>n</i> = 13
Imidazolone content (arbitrary units)			
Kidney	0.26 ± 0.02	0.31 ± 0.01	0.40 ± 0.02**
Serum			
3-DG (μM)	1.04 ± 0.08	1.23 ± 0.13	3.46 ± 0.23**
Glucose (mM)	8.33 ± 0.39	7.66 ± 0.28	31.4 ± 0.88**
Fructosamine (μM)	149 ± 2	149 ± 2	310 ± 6**
Creatinine (mM)	46.9 ± 0.2	48.6 ± 0.2	62.8 ± 7.1
Creatinine clearance (ml/s)	0.019 ± 0.001**	0.026 ± 0.001	0.018 ± 0.002**
Urine protein (mg/day)	11.3 ± 2.9	8.0 ± 0.7	63.6 ± 7.1**
Body weight (g)	228 ± 2**	403 ± 7	197 ± 5**

All results are expressed as mean ± S.E.

\**P* < 0.05, \*\**P* < 0.01 as compared with control (3 months) by Student's *t*-test.

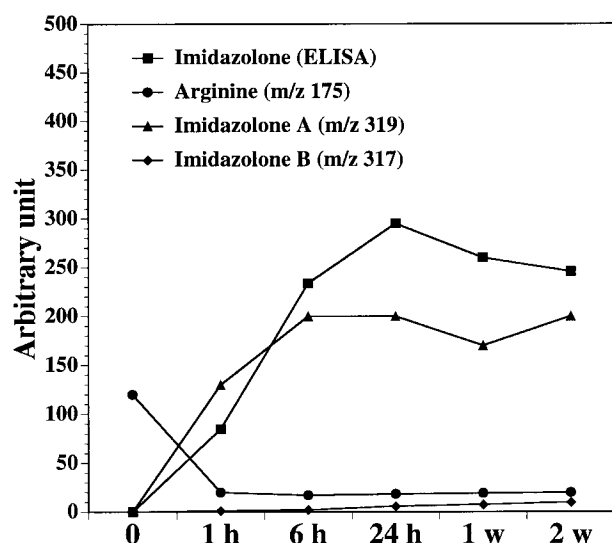


Fig. 6. Time course of the production of imidazolones as demonstrated by ELISA and LC/MS.

may lead to the increased modification of the extracellular matrix proteins with pyrraline in diabetic glomerulosclerosis. We first demonstrated that imidazolone, a chemically-identified novel AGE, was present at high levels in the kidneys of diabetic rats. Since the direct precursor of imidazolone is 3-DG but not the other dicarbonyl compounds (glyoxal or methylglyoxal), the elevated serum levels of 3-DG in diabetes result in the increased modification of extracellular matrix proteins in the kidneys with imidazolone. Thus, this study provides conclusive evidence that 3-DG is involved in the AGE modification of tissue proteins in diabetic kidneys.

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