Increased Production of Urea Hydrogen Peroxide from Maillard Reaction and a UHP-Fenton Pathway Related to Glycoxidation Damage in Chronic Renal Failure

AKIRA MOH,* NORIYUKI SAKATA,† SHIGEO TAKEBAYASHI,† KAYOKO TATEISHI,¶ RYOJI NAGAI,‡ SEIKOH HORIUCHI,‡ and JUNICHI CHIHARA§

*Department of Pathology, Medical School, Yale University, New Haven, Connecticut; †Department of Pathology, ¶Department of Biochemistry, Fukuoka University Medical School, Fukuoka, Japan; ‡2nd Department of Biochemistry, Kumamoto University School of Medicine, Kumamoto, Japan; and §Department of Internal Medicine, Tagawa City Hospital, Fukuoka, Japan.

Abstract. Urea hydrogen peroxide (UHP) is a stable form of \( \text{H}_2\text{O}_2 \) and cytotoxic agent. This study describes examination of UHP formation from collagen glycation and relevant glycoxidative damage in chronic renal failure (CRF). Renal fibers were incubated with 50 mM ribose in either serum ultrafiltrate or phosphate-buffered saline in the presence of various concentrations of urea. UHP was determined by a modified ferrous oxidation in xylenol orange (FOX) assay. The presence of urea resulted in an increase in the generation of UHP in a dose-dependent manner of urea in these incubation systems. Pentosidine levels analyzed by HPLC also increased in a dose-dependent manner of urea. Blocking experiments showed that pentosidine and carboxymethyllysine formation was significantly enhanced by hydroxyl radical generated from UHP via Fenton reaction. The renal and cardiac levels of UHP, pentosidine, and carboxymethyllysine in patients with CRF, including seven predialysis and eight hemodialysis subjects, were significantly higher than that in controls \( (n=16) \). The renal and cardiac levels of UHP closely correlated with the levels of renal and cardiac pentosidine and carboxymethyllysine and inversely correlated with left ventricle ejection fraction in CRF patients. This study provides evidence, for the first time, that UHP can be produced from Maillard reaction. Increased UHP in chronic renal failure enhances the formation of pentosidine and carboxymethyllysine via Fenton reaction (UHP-Fenton pathway).

Reducing sugar such as glucose or ribose can spontaneously react with a free amino group of proteins to form a ketoaminic group. The ketoaminic group may undergo further rearrangements and produce more stable, irreversible, and fluorescence compounds called advanced glycation end products (AGE) (1). This process is called Maillard reaction or non-enzymatic glycation. Among the molecular structures of AGE, pentosidine and carboxymethyllysine (CML) (2,3) are known to be glycoxidation products because oxidation accelerates their formation. AGE elicit a wide range of cell-mediated responses leading to vascular dysfunction, matrix expansion, and atherosclerosis (reviewed in references 4 and 5). AGE have been implicated in the pathogenesis of diabetic complications and aging (4–7).

A uremic state has been shown to be related to an increased level of pentosidine (8–9). Several studies have demonstrated a marked increase in pentosidine in the plasma proteins (10), \( \beta_2 \)-microglobulin, amyloid fibrils (11), and skin collagen (12) in patients with chronic renal failure (CRF). CML has also been shown to increase in the plasma of patients with CRF undergoing hemodialysis (13). Our immunohistochemical study showed that CML accumulated in the aortas of patients with CRF in proportion to the increasing duration of hemodialysis (14). However, the mechanism of accelerated glycoxidation in circulating or extracellular matrix proteins in CRF patients is still poorly understood. Studies aimed at elucidating the accelerated formation of pentosidine were highlighted by a study reporting that an increase in the carbonyl compounds might act as precursors of pentosidine (15).

Based on our earlier study showing increased oxidation-related fluorescence products in hemodialysis patients (16), we hypothesize that there might be a common oxidative factor(s) involved in the acceleration of glycoxidation reaction in CRF. We focused our study on reactive oxygen species related to uremic state. In the pathway of Amadori product degradation of the Maillard reaction, \( \text{H}_2\text{O}_2 \) can be generated via both 1,2- and 2,3-enolization and the oxidation of the enolate anion (17). Superoxidative products can also be formed in the pathway of Amadori product formation (17,18). \( \text{H}_2\text{O}_2 \) is unstable, easily subjected to degenerate to \( \text{H}_2\text{O} \) and \( \text{O}_2 \), and difficult to monitor in long-term incubation. In contrast, urea hydrogen peroxide...
(UHP), or carbamide peroxide in its obsolete name, is a stable form of H₂O₂ and a potential cytotoxic agent (19). The aims of the present work were to examine UHP and pentosidine formation in the context of the Maillard reaction both in vitro and in pre-hemodialysis (pre-HD) and hemodialysis patients (HD) with chronic renal failure.

Materials and Methods

Subjects

Renal and cardiac samples were obtained from 31 autopsied subjects. The clinical characteristics of these subjects are shown in Table 1. CRF in the Pre-HD patients was attributed to multiple myeloma in three patients, glomerulonephritis in two patients, rheumatoid arthritis in one patient, and hypertension in one patient. Causes of HD were glomerulonephritis in three patients, rheumatoid arthritis in two patients, hypertension in two patients, and multiple myeloma in one patient. Direct causes of death in control group were malignant tumors and hypertension complications. Subjects with diabetes mellitus were not included. The normal level of serum urea is 3.3 to 6.6 mM (MW of urea = 60; therefore, 0.198 to 0.396 mg/ml and 19.8 to 39.6 mg/dl, BUN 10 to 20 mg/dl equivalent). The mean levels of serum urea were 0.91 and 1.31 mg/ml (91.04 to 130.76 mg/dl) in pre-HD and HD group, respectively (Table 1). These levels were representative for nondiabetic pre-HD patients and HD patients in Japan. The study complies with the Declaration of Helsinki. The research protocol is approved by the ethics committee of Fukuoka University, and the informed consent of the subject’s relatives is obtained.

Reagents

Calf tendon collagen, collagenase, hydroperoxides [5(S),6E,8Z,11Z,14Z]-5-hydroperoxycosatetraenoic acid (5-HPETE), ribose, ultra-pure urea, UHP, and xylene orange were all purchased from the Sigma Chemical Co. (St. Louis). All other chemicals and reagents used were of analytical reagent grade.

Table 1. Profile of the subjects examined in this study

<table>
<thead>
<tr>
<th>Status</th>
<th>Control n = 16</th>
<th>Pre-HD n = 7</th>
<th>HD n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>61.8 ± 4.2</td>
<td>60.9 ± 6.6</td>
<td>62.5 ± 4.5</td>
</tr>
<tr>
<td>Male/female</td>
<td>9/7</td>
<td>4/3</td>
<td>5/3</td>
</tr>
<tr>
<td>Smoking (yes/no)</td>
<td>5/11</td>
<td>2/5</td>
<td>2/6</td>
</tr>
<tr>
<td>ACE inhibitor (yes/no)</td>
<td>4/12</td>
<td>2/5</td>
<td>4/4</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>11.4 ± 2.1</td>
<td>10.2 ± 1.8</td>
<td>9.7 ± 2.8</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.42 ± 0.12</td>
<td>0.51 ± 0.17</td>
<td>0.59 ± 0.26</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>12.67 ± 3.62</td>
<td>45.98 ± 10.93b</td>
<td>66.04 ± 14.30b</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>25.09 ± 7.18</td>
<td>91.04 ± 21.64b</td>
<td>130.76 ± 28.31b</td>
</tr>
<tr>
<td>Serum Cr (mg/dl)</td>
<td>1.08 ± 0.16</td>
<td>5.87 ± 2.14b</td>
<td>9.30 ± 2.88b</td>
</tr>
<tr>
<td>FBS (mg/dl)</td>
<td>104.50 ± 10.63</td>
<td>108.33 ± 17.43b</td>
<td>104.42 ± 27.47b</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3 ± 1.2</td>
<td>4.9 ± 1.6</td>
<td>4.8 ± 1.9</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>54.1 ± 15.7</td>
<td>36.9 ± 12.4</td>
<td>33 ± 14.6</td>
</tr>
<tr>
<td>Duration of hemodialysis (y)</td>
<td>–</td>
<td>–</td>
<td>6.24 ± 4.65</td>
</tr>
</tbody>
</table>

* The data were expressed as the mean ± SD.

b p < 0.01, control versus Pre-HD or HD. Left ventricle ejection fraction (LVEF) and serum concentrations of fasting blood sugar (FBS), BUN (blood urea nitrogen), and Cr (creatinine) were the average values of three measurements before a routine hemodialysis administration within the last month before death. Normal LVEF was >67%. CRP: C-reactive protein.
adding urea to a pooled sample of ultrafiltrates with concentration a little bit below the desired concentration. Typically, ultrafiltrates with concentrations of 1.0, 1.2, and 1.4 mg/ml of urea were pooled to obtain a 10 ml solution of 1.2 mg/ml of urea. For 1.4 mg/ml, 2 mg of urea was added to 10 ml containing the pooled ultrafiltrate to prepare a final urea concentration of 1.4 mg/ml. Serum ultrafiltrate from healthy subjects was used to prepare 0.4 mg/ml of urea in the similar way.

Incubation Conditions

20 mg of renal or cardiac collagen fibers prepared as described above were incubated with 50 mM ribose either in 2 ml of 0.1 M PBS (pH 7.3) or in 2 ml of the ultrafiltrates containing 0.4, 0.8, or 1.4 mg/ml of urea in sterile tubes at 37°C under aerobic condition. Toluene and chloroform (2 μl/ml each) were added to prevent microbial growth. For blocking experiments, selected incubations were performed in the presence/absence of 100 mM mannitol or in the presence of active or heat-inactivated (at 60°C) catalase (both 500 U/ml). The enzyme was put in a dialysis tube (MWCO: 8000) and replaced by a newly prepared one once a week. After incubation, each tube was centrifuged at 1500 × g for 15 min, and 1 ml of the supernatant was taken for the measurement of UHP. Then, the collagen fibers were removed from each tube, washed, and dialyzed in 0.1 M PBS, pH 7.3. The fibers were lyophilized and stored at −40°C until assay.

For molecular studies, 50 mM of arginine, lysine, and ribose were mixed in 2 ml of 0.1 M PBS (pH 7.3) at 37°C in the presence or absence of Fe²⁺/ UHP under aerobic conditions. The tubes were removed after incubation for 60 min, and 100 μl of the reaction solution was transferred to 1 ml of 6 N HCl to stop the reaction. Pentosidine levels were determined by HPLC after neutralization with 5 N NaOH.

Determination of UHP

Either 1 ml of the supernatant from the in vitro incubation (see incubation conditions) or 1 g of kidney or heart pulverized under liquid nitrogen was put into a dialysis tube (MWCO: 300; Spectrum Medical Industries, Inc., Houston, TX) to extract UHP. The tubes were suspended in 10 ml of 0.1 M PBS (pH 7.3) and stirred for 2 h at 2°C. One milliliter of the dialysates were collected and transferred to a glass tube with rough surface. The tubes were vortexed extensively for 1 min. This procedure decomposed a free form of H₂O₂ (20), but not UHP, the stable form. We tested the stability of UHP in PBS and ultrafiltrate after the vortex procedure, and the results indicated that UHP was stable in both PBS and ultrafiltrate. The dialysates thus prepared were assayed for UHP through determination of H₂O₂ released from UHP in acidic condition by ferrous oxidation in xylene orange (FOX) assay (21). The FOX assay was modified by adding horseradish peroxidase (HRP) to the reaction mixture to ensure specificity (termed HRP-FOX assay in this report). Briefly, to each tube containing the dialysates and various concentrations of UHP were added 900 μl of the reaction mixture (100 μM xylene orange, 250 μM ammonium iron [II] sulfate, 20 nM HRP, and 100 mM sorbitol in 25 mM H₂SO₄). The tubes were incubated in 37°C for 20 min, and absorbance was read at 560 nm. Calibration was performed by fitting the absorbance (492 nm) of the samples to the standard curve obtained using CML solutions of known concentrations. The CML contents were expressed as ng of CML per mg of collagen (ng/mg collagen).

Hydrolysis and Hydroxyproline Assay

Either 100 μl of the collagenase solubilized collagen or 5 mg of the freeze-dried renal/cardiac collagen fibers from incubation were acid-hydrolyzed in 2 ml of 6 N hydrochloric acid for 24 h at 110°C in a sealed glass tube. Hydroxyproline in the hydrolysate was measured according to the method of Stegemann and Stalder (22). The determination of the collagen content was based on the assumption that hydroxyproline constitutes 14% of collagen.

Pentosidine Measurement by HPLC

The hydrolysates were diluted with distilled water to an equal concentration of 2 mg/ml before an analysis by reverse-phase HPLC (23). Mobile phase A consisted of 140 mM sodium acetate in aqueous solution with 17 mM TEA titrated to pH 5.05. Mobile phase B was 60% acetonitrile. For analytical and cleaning purposes, a gradient of segments linear of solution B was programmed and performed on the system controller (Gradient conditions: initial = 0% B, 0.5 min = 2% B, 15 min = 7% B, 19 min = 13% B, 33 min = 32% B) followed by a wash with 100% B for 10 min and re-equilibration for 10 min at 100% A). The peak of pentosidine (ex 335 nm/em 385 nm, 9.8 min) was identified by spiking the peak with that of authentic pentosidine. Calibration was performed by comparing the peak areas of the samples to those of the authentic pentosidine, which was confirmed by GC/MS.

Determination of CML by ELISA

CML levels were measured by a competitive ELISA system (24). Briefly, the monoclonal antibody against CML, called 6D12, was prepared by immunizing BALB/c mice with AGE-BSA as described previously (7, 25). Peroxidase-conjugated rabbit anti-mouse IgG antibody was purchased from Organon Teknika Corp. (Westchester, PA). The samples of collagenase-extracted collagen were pretreated with 40 mM EDTA before assay. The CML concentrations of the samples were determined by fitting the absorbance (492 nm) of the samples to the standard curve obtained using CML solutions of known concentrations. The CML contents were expressed as ng of CML per mg of collagen.

Statistical Analyses

All numerical data were expressed as the mean values ± SD. The significance of chronological changes of in vitro experiment was analyzed by ANOVA. The significance of the differences between controls and subjects with renal failure was analyzed by the unpaired t test. Probability values of less than 0.05 were considered significant.

Results

Control Studies

The specificity of our HRP-FOX assay is shown in Figure 1a. Among those tested with negative results are [9(S),10E,12Z]-9-hydroperoxyoctadecadien-1-oic acid (9-HPODE), and [9Z,11E,13(S)]-9-hydroperoxyoctadecadien-1-oic acid (13-HPODE) (not shown). As shown in Figure 1b, UHP was formed only in the reaction system of ribose and collagen in the presence of urea (U+R+C). The other control combinations of the reaction system (urea and collagen, C+U; urea and ribose, R+U) did not result in UHP formation. UHP thus formed enhanced pentosidine formation when the UHP containing supernatant of reaction system (U+R+C) at 16 wk was taken to incubate with native collagen for another 16 wk.
Similar results were also obtained when ribose was replaced with glucose (not shown).

**Experiments In Vitro**

Figure 2 summarizes UHP formation during the incubation of human renal cortex collagen with 50 mM ribose in the presence of either serum ultrafiltrate containing 0.4, 0.8, and 1.4 mg/ml of urea (Figure 2a) or PBS solution containing the same amounts of ultra-pure urea (Figure 2b). These two incubation systems showed that UHP was formed in proportion to the amounts of urea at 4 and 8 wk of incubation. The pentosidine formation in this incubation system is shown in Figure 3. Both pure urea (Figure 3b) and serum ultrafiltrate (Figure 3a) from HD patients increased the pentosidine level in the cortex collagen in a urea dose-dependent manner at 8 wk of incubation. The increase in pentosidine formation could be inhibited by catalase (Figure 3, a and b). Similar results were obtained when renal cortex collagen was replaced by myocardial collagen fibers in the incubation system as described above (data not shown). In the incubations, urea concentrations were adjusted to 0.4, 0.8, and 1.4 mg/ml (40, 80, 140 mg/dl) to match the range in the patients. In fact, incubations with urea concentrations up to 200 mg/ml demonstrated increased UHP and pentosidine formation (data not shown).

The data of blocking studies in the long-term incubations show that catalase blocked both UHP and glycoxidation products (pentosidine and CML), mannitol blocked only glycoxidation products (pentosidine and CML) but not UHP, and SOD had no effect (Table 2). The results of short-term incubation (Figure 4) confirmed the effect of catalase (Figure 4j) and mannitol (Figure 4k) and elucidated that UHP enhanced pentosidine formation only in the co-presence of Fe$^{2+}$ and UHP (Fenton reaction) (Figure 4, f–h).

**UHP, Pentosidine, and CML in Renal and Cardiac Tissue of Control, Pre-HD, and HD Subjects**

The levels of UHP and pentosidine in renal and myocardial tissue are summarized in Figures 5 and 6. UHP and pentosidine...
levels in Pre-HD or HD subjects were significantly higher than in the control subjects. Similarly, the levels of CML in Pre-HD and HD subjects were significantly higher than the control subjects (Table 3).

**Correlation**

The levels of tissue UHP in Pre-HD and HD subjects closely correlated to the levels of pentosidine (Pre-HD, n = 7; renal cortex, r = 0.757; renal medulla, r = 0.764; anterior wall of myocardium, r = 0.785; posterior wall of myocardium, r = 0.781; HD, n = 8; renal cortex, r = 0.711; renal medulla, r = 0.714; anterior wall of myocardium, r = 0.728; posterior wall of myocardium, r = 0.722; all P < 0.05). The levels of tissue UHP and pentosidine inversely correlated with left ventricle ejection fraction (LVEF) of the heart (Pre-HD, n = 7. UHP versus LVEF: anterior, r = −0.765; posterior, r = −0.768. Pentosidine versus LVEF: anterior, r = −0.758; posterior, r = −0.760. HD, n = 8. UHP versus LVEF: anterior, r = −0.716, P < 0.01; posterior, r = −0.718. Pentosidine versus LVEF: anterior, r = −0.728; posterior: r = −0.726. All P < 0.05). There are also similar correlations between CML and UHP or LVEF in CRF patients (data not shown).

**Discussion**

The original FOX assay was used to measure H$_2$O$_2$ and other hydroperoxides. We added three procedures to the original FOX assay to detect H$_2$O$_2$ in UHP specifically: (1) decompose preexisted H$_2$O$_2$ by vortex; (2) cut other hydroperoxides with a dialysis membrane before an assay (most of those derived from lipid peroxidation with a molecular weight > 300); and (3) then specifically detect H$_2$O$_2$ released from UHP under HRP catalyzing (see Materials and Methods, Determination of UHP). As tested, preexisted H$_2$O$_2$ or 5-HPETE (MW: 336.5), a lipid peroxidation product with structure of ROOH, were negative in our HRP-FOX assay (Figure 1a).

To elucidate the mechanism of pentosidine formation in patients with CRF, we examined the high rate of glycation by 50 mM ribose, where the time course could be completely

---

**Table 2.** UHP and pentosidine formation during incubation of renal cortex collagen with 50 mM ribose and 1.4 mg/ml of urea in 0.1 M PBS (pH 7.4) in the presence of scavengers of reactive oxygen species at 8 wk.

<table>
<thead>
<tr>
<th></th>
<th>SOD* (inactivated)</th>
<th>SOD (500 U/ml)</th>
<th>Catalase* (inactivated)</th>
<th>Catalase (500 U/ml)</th>
<th>Mannitol (−) (0 mM)</th>
<th>Mannitol (+) (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHP (µM)</td>
<td>0.43 ± 0.05</td>
<td>0.42 ± 0.05</td>
<td>0.42 ± 0.04</td>
<td>0.33 ± 0.03</td>
<td>0.41 ± 0.04</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Pentosidine (pmol/mg)</td>
<td>129.1 ± 15.4</td>
<td>126.4 ± 12.8</td>
<td>128.7 ± 14.7</td>
<td>101.2 ± 9.6</td>
<td>127.6 ± 17.4</td>
<td>73.9 ± 7.9</td>
</tr>
<tr>
<td>CML (ng/mg)</td>
<td>252.7 ± 23.2</td>
<td>249.1 ± 22.9</td>
<td>251.3 ± 23.7</td>
<td>198.5 ± 20.2</td>
<td>247.6 ± 21.5</td>
<td>128.7 ± 15.4</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three experiments. “SOD*” and “Catalase*”: heat-inactivated SOD and catalase (500 U/ml at 60°C for 30 minutes), respectively.

* P < 0.05 between Catalase* and Catalase.

b P < 0.01 between Mannitol (−) and Mannitol (+).

---

**Figure 3.** Pentosidine formation during the incubation of renal cortex collagen with 50 mM ribose in the presence of either serum ultrafiltrate containing 0.4, 0.8, and 1.4 mg/ml of urea (a) or PBS solution containing the same amounts of ultra-pure urea (b). Data were expressed as mean ± SD of three experiments. ○, 0.4 mg/ml urea; ▲, 0.8 mg/ml urea; ▲, 1.4 mg/ml urea in the presence of UHP inhibitor (500 U/ml of catalase). ** P < 0.01 between ▲ and ○ or △ at 8 wk. * P < 0.05 between ▲ and ○ or △ at 4 wk and between ▲ and the other three incubations at 8 wk.
observed and quantitated (26). We started incubation with commercially available calf tendon collagen, which has a lower level of glycation and higher level of “native” collagen compared with aged human collagen. The results from incubation of calf tendon collagen showing that UHP formed only in the Maillard reaction in the presence of urea, but not in the absence of Maillard reaction per se (Figure 1b), clearly indicate that UHP was a Maillard reaction product of collagen and reducing sugar. UHP thus formed enhanced the pentosidine formation (Figure 1c). In the incubation of human collagen, the results show that production of UHP and pentosidine from the Maillard reaction of renal collagen was enhanced in the presence of urea in a dose-dependent manner (Figures 2 and 3). Incubation in PBS was designed as a reference for the ultrafiltrate in the present study because ultrafiltrate was a solution with complicate components, some of which were unknown.

![Figure 4](image)

**Figure 4.** Mechanism of involvement of urea hydrogen peroxide (UHP) in pentosidine formation. Pentosidine precursors (arginine, lysine, and ribose [all at 50 mM]) were incubated with various concentrations of UHP in the absence (c–e) or presence (f–h) of FeCl₂ at 37°C for 1 h. In addition, selected incubations were performed in the presence of SOD (i), catalase (j), and hydroxyl radical scavenger mannitol (k). Data were expressed as mean ± SD of three experiments. * P < 0.01, between f and a–e or g–i. ** P < 0.01, between g and both h and i. # P < 0.01, between h and j or k.

![Figure 5](image)

**Figure 5.** The levels of urea hydrogen peroxide (UHP) in renal (a) and myocardial tissue (b). Data were expressed as mean ± SD. ** P < 0.01 between control and both Pre-HD and HD.
The results in these two systems were similar and it could be concluded that the increase in UHP and pentosidine was due to the increased urea concentration, other than unknown uremic toxins in the ultrafiltrates.

Our data from blocking studies in long-term incubations showed that catalase blocked both UHP and glycoxidation products, pentosidine and CML, while mannitol blocked only glycoxidation products but not UHP, and SOD had no effect (Table 2). These data clearly implicate an involvement of UHP and hydroxyl radical in the enhanced glycoxidation. These results agree with an earlier report showing enhanced formation of CML by hydroxyl radical (27). However, the blocking rate of catalase (about 20%) was low, probably due to inactivation of catalase in long-term incubation. To confirm and elucidate the mechanism, we designed a short-term incubation system and the result of catalase blocking under short incubation (Figure 4j) is similar to that from experiments conducted under long-term incubation but with a higher response than the long-term one (Table 2). Incubation of arginine, lysine, and ribose (pentosidine precursors) with various concentrations of UHP in Fe$^{2+}$-free medium was not associated with enhanced pentosidine formation (Figure 4, c–e). Although UHP contains a potential reactive oxygen species, H$_2$O$_2$, this stable form of oxidative chemical does not directly enhance pentosidine formation. Fe$^{2+}$ alone in the absence of UHP did not increase pentosidine formation either (Figure 4b). In contrast to the reaction in the presence of either iron or UHP alone, introduction of Fenton reaction to the reaction system to produce hydroxyl radical by adding Fe$^{2+}$ to the reaction mixture enhanced pentosidine formation in a UHP dose-dependent manner (Figure 4, f–h). The enhanced formation was significantly inhibited by either hydroxyl radical scavenger mannitol or H$_2$O$_2$ scavenger catalase (Figure 4, j and k). These data provided evidence for the presence of a UHP-Fenton pathway for

**Figure 6.** The levels of pentosidine in renal cortex and medulla (a) and cardiac collagen of the anterior and posterior wall of the left ventricle (b). Data were expressed as mean ± SD. **P < 0.01 between control and HD; * P < 0.05 between Pre-HD and both control and HD.

**Table 3.** CML levels (ng/mg) obtained from renal and myocardial collagen in control, Pre-HD, and HD groups

<table>
<thead>
<tr>
<th>Location</th>
<th>Control (n = 16)</th>
<th>Pre-HD (n = 7)</th>
<th>HD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>62 ± 18</td>
<td>107 ± 19*</td>
<td>147 ± 19b</td>
</tr>
<tr>
<td>Medulla</td>
<td>70 ± 20</td>
<td>119 ± 23a</td>
<td>158 ± 23b</td>
</tr>
<tr>
<td>Myocardium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>122 ± 20</td>
<td>187 ± 24a</td>
<td>272 ± 28b</td>
</tr>
<tr>
<td>Posterior</td>
<td>114 ± 23</td>
<td>174 ± 23a</td>
<td>254 ± 27b</td>
</tr>
</tbody>
</table>

* P < 0.05 between Pre-HD and both control and HD.

b P < 0.01 between HD and control.

The results in these two systems were similar and it could be concluded that the increase in UHP and pentosidine was due to the increased urea concentration, other than unknown uremic toxins in the ultrafiltrates.
the accelerated glycooxidation in chronic renal failure in vitro. The outline of the UHP-Fenton pathway is demonstrated in Figure 7.

In vivo, the increased UHP in CRF patients (Figure 5) could be produced by two steps. First, $H_2O_2$, from which UHP was formed, could be generated during the formation and degradation of Amadori products derived from reducing sugar such as glucose (17,18). Second, high level of urea shifted the reversible reaction between $H_2O_2$ and urea toward the formation of stable UHP, which accumulates in tissue of CRF patients. We think that both high level of urea and increased Amadori products in CRF (28) in these two steps may contribute to the increase in UHP level in CRF.

In vitro, our results of increased glycooxidation in the incubation of collagen (Figure 3) suggest that trace levels of iron in the phosphate (70 μg/dl according to the manufacturer; normal level of serum iron is 60 μg, approximately 150 μg/dl.) was sufficient in the in vitro collagen incubation system to account for the accelerated formation of glycooxidation products. In vivo, it is well known that CRF is associate with a bleeding tendency and hence an overexposure of extracellular matrix to iron. It seems that tissue iron is sufficient in the CRF patients to induce the accelerated formation of glycooxidation products. As demonstrated in Table 3 and Figures 5 and 6, the levels of UHP and glycooxidation products were markedly higher in both Pre-HD and HD groups compared with the control subjects. The UHP level closely correlated with the level of glycooxidation products (see “Correlation” in “Results”). These data provided strong implicafor the presence of UHP-Fenton pathway in subjects with chronic renal failure. In diabetes, the high level of glucose may explain the increased formation of glycooxidation products. However, such a condition as continuous hyperglycemia is not present in chronic renal failure. The presence of UHP-Fenton pathway might be an important factor for the elevated levels of glycooxidation products in chronic renal failure. In addition, we also detected a high level of serum UHP in CRF patients (data not shown). However, serum albumin itself is a strong antioxidant, and the interaction between UHP and serum proteins remains to be examined.

Both UHP and $H_2O_2$ have potential deleterious effects on various cells, including those of kidney and heart (19,29,30). In addition, glycooxidation have been shown to cause alterations in the chemical structures and functions of collagen fibers, including cross-linking, rigidity, and insolubility (31,32). For predialysis patients, UHP and pentosidine could accelerate the damage of the heart and destroying kidney. For patients under dialysis, although dialysis could partially replace the destroyed kidney, it could reduce neither UHP nor pentosidine to a normal level and the damage to the heart could be fatal. Actually, cardiac complications are now accounting for between 40 and 50% of all death in patients with CRF in a cohort (33). In this study, the increased UHP and pentosidine inversely correlated with LVEF (see results) in both Pre-HD and HD subjects. The recognition that CRF is related to the increased UHP and glycooxidation in renal and cardiac matrix is expected to manage these toxins to delay functional damage of the heart and destroying kidney for predialysis patients or to accelerate the removal by developing more effective dialysis procedures for patients under dialysis.

In summary, the present study provided evidence for the presence of a UHP-Fenton pathway for the accelerated glycooxidation in patients with CRF, and this oxidation pathway might contribute to renal and cardiac damage in patients with CRF.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (C) (2) (14570171) from The Ministry of Education, Culture, Sports, Science and Technology (MEXT) and funds from Central Research Institute of Fukuoka University and Foundation for Promotion of Research on Clinical Medicine.

**Maillard reaction**

*Figure 7.* The proposed UHP-Fenton pathway for the enhanced formation of UHP and consequent increase in the production of pentosidine from the Maillard reaction in chronic renal failure. +, enhancement; †, increase.
References


