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Hypochlorous acid generates N^{ε}-(carboxymethyl)lysine from Amadori products

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Abstract

Since the accumulation of N^{ε}-(carboxymethyl)lysine (CML), a major antigenic advanced glycation end product, is implicated in tissue disorders in hyperglycemia and inflammation, the identification of the pathway of CML formation will provide important information regarding the development of potential therapeutic strategies for these complications. The present study was designed to measure the effect of hypochlorous acid (HOCl) on CML formation from Amadori products. The incubation of glycated human serum albumin (glycated-HSA), a model of Amadori products, with HOCl led to CML formation, and an increasing HOCl concentration and decreasing pH, which mimics the formation of these products in inflammatory lesions. CML formation was also observed when glycated-HSA was incubated with activated neutrophils, and was completely inhibited in the presence of an HOCl scavenger. These data demonstrated that HOCl-mediated CML formation from Amadori products plays a role in CML formation and tissue damage at sites of inflammation.

Keywords: *N^{ε}-(carboxymethyl)lysine (CML), advanced glycation end products (AGEs), neutrophils, atherosclerosis*

Abbreviations: *AGE(s), advanced glycation end products; RAGE, receptor for AGE; HSA, human serum albumin; CML, N^{ε}-(carboxymethyl)lysine; HOCl, hypochlorous acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DTPA, diethylenetriamine*-N,N,N′,N″,N‴*-pentaacetic acid; PMA, phorbol 12-myristate 13-acetate*

Introduction

The long-term incubation of proteins with glucose leads, through formation of Schiff base and Amadori products, to the generation of advanced glycation end products (AGEs) of the Maillard reaction. These are characterized by a yellow–brown color, an autofluorescence, intra- and inter-molecular cross-linkings. AGE-modified proteins increase during normal process of aging, but this is markedly accelerated in diabetics with sustained hyperglycemia. The accumulation of AGE-modified proteins can lead to tissue damage and contribute to the development of diabetic complications through a variety of mechanisms: through an alteration in the structure and function of tissue proteins [1], by the stimulation of cellular responses via receptors specific for AGE-modified proteins [2,3] or by the generation of reactive oxygen intermediates [4,5].

Among the AGE structures previously characterized, N^{ε}-(carboxymethyl)lysine (CML) is the most antigenic [6,7]. CML concentration, adjusted for age and duration of diabetes, is further increased in

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patients who have severe complications, including nephropathy [8–10], retinopathy [11], and atherosclerosis [12–14]. We previously demonstrated that CML is generated by the oxidative cleavage of Amadori products by hydroxyl radical and peroxynitrite, thus suggesting CML to be an important biological markers of oxidative stress *in vivo* [15,16]. CML is recognized by receptor for AGE (RAGE), and CML–RAGE interaction activates cell signaling pathways such as NF-κB and enhances the expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells [17]. Furthermore, Alikhani et al. demonstrated that CML-collagen is recognized by RAGE and induces fibroblast apoptosis via the expression of caspase-3, -8, and -9 [18]. The AGE inhibitors aminoguanidine and pyridoxamine block CML formation and retard the development of early renal disease in the streptozotocin-diabetic rat [19]. Considered together, these studies strongly suggest an association between CML and the development of diabetic complications.

Although AGEs formation is associated with an increased glucose concentration, CML accumulation also increases in non-diabetic kidney diseases [20]. This indicates that there are other pathways, independent of the glucose concentration, for their formation. One potential mechanism involves activated phagocytes including neutrophils and macrophages. Recent immunological studies demonstrate that CML accumulate at the sites of activated phagocyte infiltration such as atherosclerotic lesions [13,21]. Anderson et al. demonstrated that the hypochlorous acid (HOCl)–serine system generates CML via production of glycolaldehyde [22,23]. HOCl is by far the most abundant agent generated by phagocytic cells and react readily with tissue proteins, thus disrupting their structure and function. Therefore, HOCl could be a primary mediator of inflammatory tissue damage. Although we also detected CML formation during the incubation of protein with glycolaldehyde, GA-pyridine was predominantly generated in this situation, rather than CML [24]. This suggests that glycolaldehyde-mediated CML formation may, therefore, be a minor pathway *in vivo*.

The present study investigates the possibility that HOCl directly reacts with Amadori products to form CML. These results suggest that HOCl-mediated CML formation from Amadori products is remarkably faster than the previously reported glycolaldehyde-mediated pathway, and it may, therefore, play an important role in CML formation and tissue damage at sites of inflammation.

Materials and methods

Materials

Human serum albumin (HSA) was donated by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted using charcoal treatment as described by Chen [25]. D-Glucose was obtained from Nacalai Tesque (Kyoto, Japan). Sodium hypochlorite and glycolaldehyde were purchased from Sigma (St Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD). All other chemicals were of the best grade available from commercial sources.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [6]. Briefly, each well of a 96-well microtiter plate was coated with 100 μl of the indicated concentration of sample in PBS, and incubated for 2 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were then blocked with 0.5% gelatin in PBS for 1 h. After washing 3 times, the wells were incubated for 1 h with 100 μl of the indicated concentration of monoclonal anti-CML antibody (6D12). After triplicate washing, the wells were incubated with HRP-conjugated anti-mouse IgG, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 μl of 1.0 M sulfuric acid, and then the absorbance was read at 492 nm with a micro-ELISA plate reader.

Determination of CML contents by HPLC

The CML contents of the samples were determined by an amino acid analysis after acid hydrolysis with 6 N HCl for 24 h at 110°C with an amino acid analyzer (L-8500A, Hitachi) (Tokyo, Japan) equipped with an ion exchange column (#2622 SC, 4.6 × 80 mm, Hitachi), as described previously [26].

Effect of HOCl on CML formation from glycated-HSA

Glycated-HSA and nonglycated-HSA were prepared as described previously [15,16]. Amino acid analysis showed that 12.5 of 59 lysine residues were modified by glucose in glycated-HSA, while its CML content was less than a detectable level (< 0.01 mol CML/mol glycated-HSA). The concentration of HOCl was measured by using a molar extinction coefficient of 350 M^{-1} cm^{-1} at 290 nm at pH 12. To determine the effect of HOCl on CML formation, glycated-HSA (2 mg/ml) was incubated with indicated concentration of HOCl at 37°C for 5 s in 50 mM sodium phosphate buffer (pH 7.4), and reaction was terminated by addition of 1/100 volume of 1.5 M sodium sulfite. CML content was measured by ELISA using a monoclonal anti-CML antibody (6D12) and HPLC after acid hydrolysis. To determine the effect of pH on CML formation, HOCl treatment of glycated-HSA was also carried out in either 50 mM sodium citrate

buffer (pH 5.0), 50 mM sodium phosphate buffer (pH 6.0) or 50 mM sodium phosphate buffer (pH 7.0).

Incubation of glycated-HSA with human neutrophils

Neutrophils were isolated from heparinized peripheral blood specimens of healthy donors using Polymorphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. The purity of the neutrophil preparations routinely exceeded 95%, and cell viability, as determined by propidium iodide staining, was at least 98%. Glycated-HSA or nonglycated-HSA (0.2 mg/ml) was added to purified neutrophil suspension (5×10^6 cells/ml) in medium A (50 mM sodium phosphate, 100 mM NaCl, 4 mM KCl, and 100 μM DTPA (pH 7.4)). Neutrophils were then activated with 250 nM phorbol 12-myristate 13-acetate (PMA) and incubated with intermittent inversion for 45 min at 37°C. Neutrophils were then removed by centrifugation (5 min 10,000*g*), and the CML content was measured by noncompetitive ELISA.

Effect of glycolaldehyde on CML formation from glycated-HSA

To determine the effect of glycolaldehyde on CML formation from glycated-HSA, glycated-HSA (2 mg/ml) was incubated with 1 or 10 mM glycolaldehyde in 50 mM sodium phosphate buffer (pH 7.4) for 5 s and then the CML content was measured by noncompetitive ELISA.

Statistical analysis

All data were expressed as the mean ± SD. Statistical significance was evaluated using ANOVA. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Effect of HOCl on CML formation from glycated-HSA

To determine whether CML could be generated from Amadori products by HOCl treatment, glycated-HSA was incubated with an increasing concentration of HOCl at 37°C for 5 s, followed by the determination of CML by noncompetitive ELISA. As shown in Figure 1A, treatment of glycated-HSA with HOCl resulted in a significant increase in CML production, in a dose dependent manner. In a time-course study, the HOCl-mediated CML formation from glycated-HSA reached a plateau as early as 5 s (data not shown). To confirm the results obtained from ELISA, CML level was measured in HOCl-treated glycated-HSA after acid hydrolysis. An HPLC analysis demonstrated that 0.44 mol CML/HSA on glycated-HSA was detected by 7.5 mM HOCl treatment.

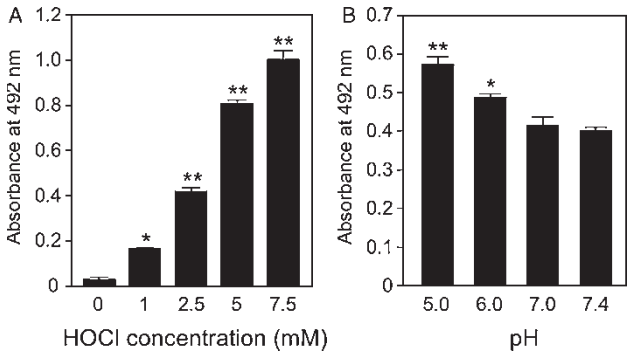

Figure 1. Effect of HOCl on CML formation from glycated-HSA. (A) Glycated-HSA (2 mg/ml) was incubated with increasing concentrations of HOCl at 37°C for 5 s in 50 mM sodium phosphate buffer (pH 7.4), followed by determination of CML by noncompetitive ELISA. HOCl-treated samples (10 μg/ml) were coated on the ELISA plate and incubated for 2 h. The wells were washed and blocked with gelatin, followed by reaction with anti-CML antibody. The antibodies bound to the wells were detected by HRP-conjugated anti-mouse IgG antibody, as described under the "Materials and methods". Data are the mean ± SD ($n = 3$). $*P < 0.01$ and $**P < 0.001$, with respect to untreated glycated-HSA. (B) Glycated-HSA (2 mg/ml) was incubated with 2.5 mM HOCl at 37°C for 5 s with decreasing pH; 50 mM sodium phosphate buffer for pH 6.0–7.4 and 50 mM sodium citrate buffer for pH 5.0. The CML content was determined by noncompetitive ELISA as described above. Data are the mean ± SD ($n = 3$). $*P < 0.01$ and $**P < 0.001$, with respect to pH 7.4.

In contrast, CML content was below detectable level (< 0.01 mol CML/mol HSA) when nonglycated-HSA was also incubated with HOCl, indicating that Amadori products were converted into CML by HOCl treatment. To determine the effect of pH on CML formation, glycated-HSA was also incubated with HOCl for 5 s at decreasing pH, followed by determination of CML by noncompetitive ELSIA. As shown in Figure 1B, the CML formation from glycated-HSA increased as the pH decreased.

Incubation of glycated-HSA with human neutrophils

Activated phagocytes such as neutrophils, are a major source of HOCl production and play a fundamental role in host defense. To determine whether activated human neutrophils also generate CML, glycated-HSA or nonglycated-HSA was incubated with PMA-activated neutrophils, followed by the determination of CML by noncompetitive ELISA. As shown in Figure 2, a significant amount of CML was generated when glycated-HSA was incubated with PMA-activated neutrophils, whereas the CML level was remained at background levels in the experiment with nonglycated-HSA. Furthermore, the activated neutrophils-mediated CML formation from glycated-HSA was completely inhibited in the presence of methionine, an HOCl scavenger. These results suggest that the HOCl-mediated CML formation

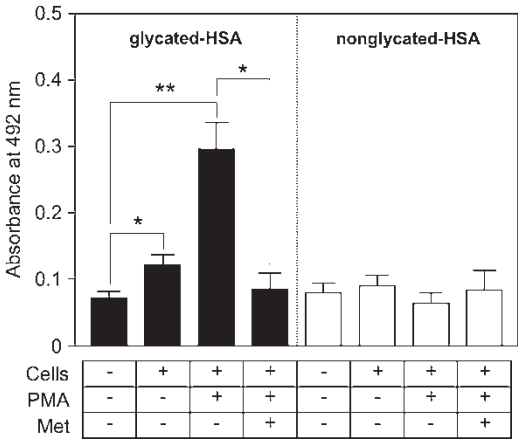

Figure 2. Effect of activated neutrophils on CML formation from glycated-HSA. Glycated-HSA and nonglycated-HSA (0.2 mg/ml) were incubated with PMA-activated neutrophils at 37°C for 45 min in the presence of 100 μM DTPA. The HOCl scavenger methionine (Met; 1 mM) was added 5 min prior to PMA addition. Following incubation, the neutrophils were removed by centrifugation (10,000*g* for 5 min), and the CML content was measured by noncompetitive ELISA. The samples (10 μg/ml) were coated on the ELISA plate and then were incubated for 1 h. The wells were washed and blocked with gelatin, followed by reaction for 1 h with anti-CML antibody. The antibodies bound to wells were detected by HRP-conjugated anti-mouse IgG antibody. The data are the mean ± SD ($n = 4$). $\star P < 0.05$; $\star\star P < 0.01$.

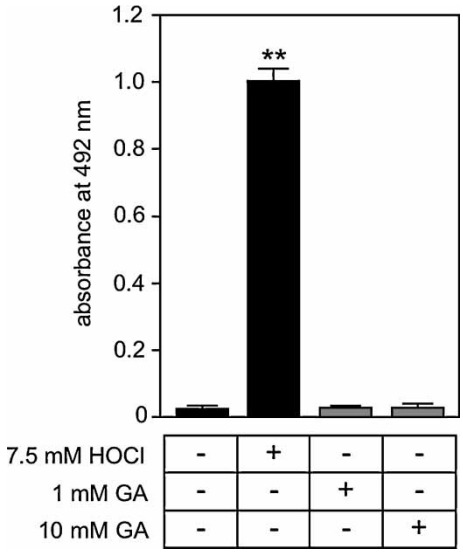

Figure 3. Effect of glycolaldehyde on CML formation from glycated-HSA. Glycated-HSA was incubated at 37°C for 5 s with 7.5 mM HOCl or 1 and 10 mM glycolaldehyde (GA) followed by determination of CML by noncompetitive ELISA. HOCl-treated or GA-treated samples (10 μg/ml) were coated on the ELISA plate and CML was detected as described Figure 1. Data are the mean ± SD ($n = 3$). $\star\star P < 0.001$, with respect to untreated glycated-HSA.

from Amadori products may, therefore, play a role *in vivo*.

Effect of glycolaldehyde on CML formation from glycated-HSA

Anderson et al. reported that the HOCl–serine system generates CML via production of glycolaldehyde (glycolaldehyde pathway) [22,23]. To determine the possible involvement of glycolaldehyde as an intermediate during HOCl-induced CML formation, glycated-HSA was incubated with glycolaldehyde at 37°C for 5 s, followed by determination of CML by noncompetitive ELISA. As shown in Figure 3, a significant amount of CML was generated when glycated-HSA was incubated with HOCl, whereas CML remained below detectable levels in glycolaldehyde-treated glycated-HSA, thus indicating that the HOCl-mediated CML formation from glycated-HSA was, therefore, independent from the glycolaldehyde pathway.

Discussion

The myeloperoxidase system in phagocytes such as neutrophils and activated macrophages generates HOCl that kills invading bacteria and viruses. However, an excess amount of HOCl produced by inflammatory responses has been implicated in the damage of tissues under pathological conditions. In the present study, a significant amount of CML was generated when glycated-HSA was incubated with HOCl for 5 s, and increased with either increasing HOCl concentration or lowering pH, which mimics the conditions in inflammatory lesions. The HOCl-mediated CML formation from Amadori products was also determined by HPLC analysis, whereas CML was under a detectable level when nonglycated-HSA was incubated with HOCl. HOCl-induced CML formation was faster than that generated by the glycolaldehyde-mediated pathway [22]. Furthermore, CML formation was also observed when glycated-HSA was incubated with PMA-activated neutrophils, indicating that HOCl-induced CML formation from Amadori products may play a role in CML formation in inflammatory lesions (Figure 4).

Activated neutrophils generate superoxide anion radical and its derivatives such as hydrogen peroxide, hydroxyl radical and singlet oxygen via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, and HOCl from the myeloperoxidase system in the presence of chloride and hydrogen peroxide. Previous research demonstrated that CML is generated by oxidative cleavage of Amadori products by metal-catalyzed hydroxyl radicals, and it is significantly inhibited by metal chelators [15]. In the present study, a transition metal chelator, DTPA, was supplemented in the reaction medium to suppress the hydroxyl radical produced by the Fenton reaction. Nevertheless, incubation of glycated-HSA with activated neutrophils led to CML formation, but this was inhibited by HOCl quencher, thus indicating that neutrophils-induced CML formation of glycated-HSA

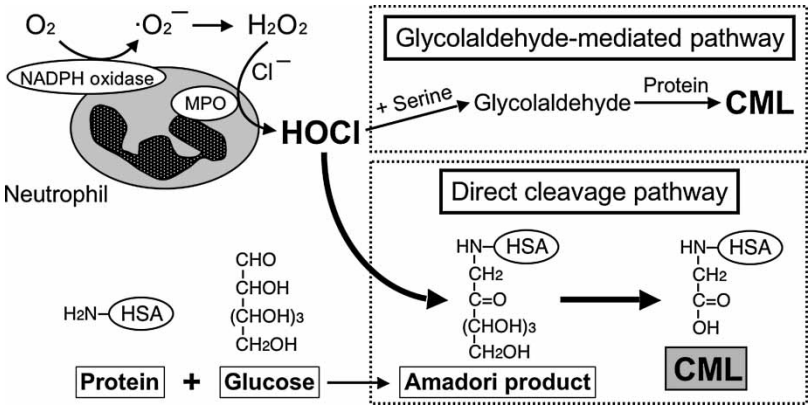

Figure 4. Proposed mechanism of CML formation by the action of HOCl.

was independent from the metal-catalyzed Fenton reaction. Myeloperoxidase is detected in lipid-laden macrophages in human atherosclerotic lesions [27]. Furthermore, Hazell et al. [28] demonstrated that HOCl-modified proteins are present in monocyte/-macrophages in human atherosclerotic lesions, indicating that myeloperoxidase is activated and plays a role in protein modification in these lesions. Recent studies, using antibodies specific for CML, demonstrated intense immunostaining of macrophages in such lesions [21]. We also found that CML-modified protein colocalized with 3-chlorotyrosine, a specific protein modification products formed by myeloperoxidase, in ruptured carotid arteries (data not shown). Moreover, CML is detected in atherosclerotic lesions in patients with end-stage renal diseases [13], and euglycemic animals [29], indicating that CML accumulation is enhanced not only under hyperglycemic conditions but also in association with inflammation involved in the myeloperoxidase system.

Anderson et al. [22] previously demonstrated the HOCl–serine system to generate CML via the production of glycolaldehyde (glycolaldehyde pathway), whereas the glycolaldehyde production from this system decreased at lower pH levels [23], which is characteristic of inflammatory lesions. The present study, however, demonstrated that HOCl-induced CML formation from Amadori products increased as the pH decreased (Figure 1B). Furthermore, the previous study demonstrated that although 83% of lysine residues are modified during incubation of BSA with 33 mM glycolaldehyde for 7 days, only 2.5% of this modification is accounted for by the formation of CML [26]. GA-pyridine, a glycolaldehyde-derived AGE structure, is detectable after 2 h, and its level increases rapidly up to 8 h, whereas the level of CML increases slowly but progressively until 24 h [24]. Although the generation of Amadori products takes a long time after the reaction of protein with glucose, CML can be generated within a few seconds through the oxidation of Amadori products. Amadori products are generated under physiological condition, and it exists ubiquitously in almost all tissues *in vivo*. The present study clearly demonstrated that HOCl generates CML from Amadori products at 5 s, and may play an important role in CML formation *in vivo* (Figure 4).

As described in the introduction, CML is recognized by RAGE, and CML–RAGE interaction generates an inflammatory response in endothelial cells [17], and induces apoptosis in fibroblast [18]. Recent clinical studies also demonstrated the serum CML level to be a risk factor associated with mortality in patients with uremia [30]. Considered together, these studies strongly suggest an association between CML and the development of diabetic complications and age related disorders. Since the presence of CML has been demonstrated in a variety of tissue proteins, HOCl-mediated CML formation from Amadori products is potentially involved in various lesions associated with long-term oxidative damage *in vivo*. Future studies are therefore required to determine the effect of inhibitors of HOCl production or scavengers of HOCl on the progression of vascular diseases.

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