Peroxynitrite Induces Formation of N^{ε}-(Carboxymethyl)Lysine by the Cleavage of Amadori Product and Generation of Glucosone and Glyoxal From Glucose

Novel Pathways for Protein Modification by Peroxynitrite

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Accumulation of advanced glycation end products (AGEs) on tissue proteins increases with pathogenesis of diabetic complications and atherosclerosis. Here we examined the effect of peroxynitrite ($ONOO^-$) on the formation of N^{ε}-(carboxymethyl)lysine (CML), a major AGE-structure. When glycated human serum albumin (HSA; Amadori-modified protein) was incubated with $ONOO^-$, CML formation was detected by both enzyme-linked immunosorbent assay and high-performance liquid chromatography (HPLC) and increased with increasing $ONOO^-$ concentrations. CML was also formed when glucose, preincubated with $ONOO^-$, was incubated with HSA but was completely inhibited by aminoguanidine, a trapping reagent for α-oxoaldehydes. For identifying the aldehydes that contributed to $ONOO^-$-induced CML formation, glucose was incubated with $ONOO^-$ in the presence of 2,3-diaminonaphthalene. This experiment led to identification of glucosone and glyoxal by HPLC. Our results provide the first evidence that $ONOO^-$ can induce protein modification by oxidative cleavage of the Amadori product and also by generation of reactive α-oxoaldehydes from glucose.
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Long-term incubation of proteins with glucose leads, through formation of Schiff bases and Amadori products, to the generation of advanced glycation end products (AGEs), which are characterized by fluorescence, brown color, and intra- or intermolecular cross-linking of protein. Although several AGE structures have been reported, our group (1) and Reddy et al. (2) independently demonstrated that N^{ε}-(carboxymethyl)lysine (CML) is a major antigenic AGE structure. CML concentration, adjusted for age and duration of diabetes, is also increased in patients who have diabetes with complications, including nephropathy (3–5), retinopathy (6), and atherosclerosis (7–9). CML is also 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 (10). The AGE inhibitors aminoguanidine and pyridoxamine also inhibit CML formation and retard the development of early renal disease in the streptozotocin-diabetic rat (11). Considered together, these studies strongly suggest an association between CML and the development of diabetic complications.

Nitric oxide (NO), or endothelium-derived relaxing factor, exhibits several physiological functions, such as inhibition of neutrophil adhesion (12), enhancement of platelet aggregation (13), and regulation of vascular relaxation (14). Furthermore, NO is extremely reactive with superoxide anion radical (O_2^-), generating peroxynitrite ($ONOO^-$) (15), which functions as an oxidant to proteins, vitamins, and DNA (16). One hypothesis argues that the balance between NO and O_2^- generation is a critical determinant in the cause of many human diseases, including atherosclerosis, neurodegenerative diseases, ischemia-reperfusion injury, and cancer (17). NO and O_2^-, both of which can be generated by aortic endothelial cells during hyperglycemia, can disturb the functions of those cells and are known to induce vascular disorders (18). Furthermore, O_2^- generated from glycated LDL reacts with NO and decreases the NO-induced modulation of cellular cyclic GMP levels (19). These reports suggest that increased

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AGE, advanced glycation end product; CML, N^{ε}-(carboxymethyl)lysine; DAN, 2,3-diaminonaphthalene; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization–mass spectrometry; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; HSA, human serum albumin; iNOS, inducible nitric oxide synthase; KLH, keyhole limpet hemocyanin; NO, nitric oxide; O_2^-, superoxide anion radical; $ONOO^-$, peroxynitrite; RAGE, receptor for AGE; TGF-β1, transforming growth factor-β1.

generation of $ONOO^-$ as well as O_2^- may contribute to the vascular complications of diabetes. For this purpose, we studied the effects of $ONOO^-$ on formation of CML from Amadori adducts on protein (glycoxidation) and from glucose preincubated with $ONOO^-$ then exposed to protein (antioxidative glycosylation). Our results indicate that $ONOO^-$ induces CML formation not only from Amadori product but also from glucose and that, in the latter pathway, glucosone and glyoxal are intermediates in the formation of CML.

RESEARCH DESIGN AND METHODS

Chemicals. D-Glucose and human serum albumin (HSA; fraction V) were purchased from Sigma (St. Louis, MO). Diethylenetriaminepentaacetic acid, 2,3-diaminonaphthalene (DAN), 3-deoxyglucosone, and $ONOO^-$ were purchased from Dojindo Laboratories (Kumamoto, Japan). Nitrotyrosine was purchased from Cayman Chemical Company (Ann Arbor, MI). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD). Microtitration plates (96-well; Nunc Immunoplate-maxisorp) were purchased from Nippon Inter Med (Tokyo, Japan). Glyoxal was obtained from Nacalai Tesque (Kyoto, Japan), and methylglyoxal was obtained from Sigma. All other chemicals were of the best grade available from commercial sources.

Preparation of monoclonal antibody against CML and nitrotyrosine. The experimental protocol was approved by the local ethics review committee for animal experimentation. Monoclonal anti-AGE antibody 6D12 was prepared in mice using AGE-modified BSA (20) and was previously shown to recognize CML-protein adducts (1). Antigen for preparation of anti–nitrotyrosine antibody was made by coupling of nitrotyrosine to keyhole limpet hemocyanin (KLH) or HSA. Nitrotyrosine (6 mg) and protein (6 mg) were dissolved in 2.7 ml PBS, followed by 0.03 ml of 250 mmol/l *N*-hydroxysulfosuccinimide (Pierce). Coupling was initiated by addition of 0.3 ml of 500 mmol/l 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce), and the reaction was incubated at room temperature for 1 h. The reaction was terminated by addition of 3 μl of 2-mercaptoethanol to a final concentration of 20 mmol/l, and the solution was dialyzed against PBS at 4°C for 24 h. Splenic lymphocytes from Balb/c mouse immunized with nitrotyrosine-conjugated HSA (nitrotyrosine-HSA) were fused to myeloma P3U1 cells. The hybridoma cells positive to nitrotyrosine-conjugated KLH (nitrotyrosine-KLH) but negative to HSA and KLH were selected through successive subcloning. Cell line 2H1 was used to prepare ascites fluid in mice, and antibody 2H1 was further purified by affinity chromatography on protein G–immobilized Sepharose gel to IgG1.

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (21). Briefly, each well of a 96-well microtiter plate was coated with 100 μl of the sample to be tested in 50 mmol/l sodium carbonate buffer (pH 9.6), blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). Wells were incubated with 0.1 ml of 6D12 (0.1 μg/ml) or 2H1 (1 μg/ml) dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by addition of 0.1 ml of 1.0 mol/l sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader.

Detection of CML and nitrotyrosine by high-performance liquid chromatography. Authentic CML was prepared by overnight incubation of 0.26 mol/l N^{α}-acetyllysine with 0.13 mol/l glyoxylic acid in the presence of 0.65 mol/l sodium cyanoborohydride in 0.5 ml of 0.1 mol/l sodium carbonate buffer (pH 10.0) at room temperature, followed by acid hydrolysis with 6 N of HCl at 110°C for 24 h (22). The CML and nitrotyrosine content of the samples were quantified by acid hydrolysis with 6 N of HCl for 24 h at 110°C, followed by amino acid analysis on a Hitachi L-8500A instrument equipped with an ion-exchange high-performance liquid chromatography (HPLC) column (#2622 SC, 4.6 × 80 mm; Hitachi) and a ninhydrin postcolumn detection system, as described previously (21).

Effect of $ONOO^-$ on CML and nitrotyrosine formation. Glycated HSA and nonglycated HSA were prepared as described previously (23). Amino acid analyses showed that 6.5 of 59 lysine residues were modified by glucose in glycated HSA. The concentration of $ONOO^-$ was determined by absorbance at 302 nm ($\varepsilon M = 1{,}670$). For determining the effect of $ONOO^-$ on CML formation in vitro, glycated HSA or nonglycated HSA (2 mg/ml) was incubated at 37°C for 5 min with 6 mmol/l $ONOO^-$ in 50 mmol/l sodium phosphate-buffered solution (pH 7.4) in the presence of 20 mmol/l sodium hydrogen carbonate (buffer A), followed by determination of CML and nitrotyrosine content by noncompetitive ELISA and HPLC. We also determined the dose-dependent effect of $ONOO^-$ (64–640 μmol/l) on CML formation by noncompetitive ELISA under the same experimental conditions.

Effect of preincubation of $ONOO^-$ with glucose on CML formation. D-Glucose (36 mmol/l) was preincubated at 37°C for 30 min with different concentrations of $ONOO^-$ (28–1,400 μmol/l) in 1 ml of buffer A; the disappearance of $ONOO^-$ was monitored by absorbance at 302 nm. $ONOO^-$-treated glucose was further incubated at 37°C for 24 h with 1.4 mg/ml HSA, followed by determination of CML by noncompetitive ELISA.

Detection of α-oxoaldehydes using DAN. Generation of α-oxoaldehydes from $ONOO^-$-treated glucose was determined by measurement of DAN–α-oxoaldehydes adduct as described previously (24). Briefly, 142 mmol/l glucose was incubated at 37°C for 5 min with 2.6 mmol/l $ONOO^-$ in PBS and further incubated at 37°C for 1 h in the presence of DAN (0.47 mmol/l). The aldehyde-DAN adducts were analyzed by reverse-phase HPLC using a Mightysil RP-18 GP column (150 mm, 3 mm; 3-μm particle size; Kanto Chemical, Tokyo, Japan). Effluents were monitored by fluorescence (λ ex/em = 271/503 nm). All analyses were performed at 40°C at an elution rate of 0.4 ml/min using an elution buffer composed of 70% phosphoric acid (50 mmol/l), 15% methanol, and 15% acetonitrile. Glucosone (D-arabino-hexosulose) was synthesized as described previously (25). Glucosone-DAN and glyoxal-DAN adducts were prepared by incubating the respective aldehydes (0.41–100 μmol/l) with DAN (0.47 mmol/l) at 37°C for 1 h and were used to construct standard curves.

HPLC-mass spectrometry. HPLC system (Nanospace SI-1 series; Shiseido, Tokyo) equipped with electrospray ionization–mass spectrometry (ESI-MS) of LCQ MSn system (Thermo Quest, San Jose, CA) was used for the analysis of DAN-aldehyde adducts using a Mightysil RP-18 GP column for chromatographic separation. The mobile phase A was 70% acetic acid (5 mmol/l in water), 15% acetonitrile, and 15% methanol. Mobile phase B was 70% acetonitrile and 30% acetic acid (50 mmol/l in water). For analyte separation, the initial mobile phase composition was 100% mobile phase A, then programmed linearly to 0% after 30 min with a flow rate set at 0.5 ml/min. Column temperature was set at 40°C, and injection volume was 10 μl. The mass spectrometer was operated in positive ion mode using a capillary voltage of 10 V; the spray voltage was set at 5 kV. Mass spectra were collected in the full-scan mode, scanning over the range 150–350 m/z (mass-to-charge ratio). The capillary temperature was maintained at 260°C. Ions were generated using nitrogen as sheath and auxiliary gas at flow rates of 90 and 20 l/h, respectively.

RESULTS

Effect of $ONOO^-$ on CML formation from Amadori products. To determine whether CML could be generated by $ONOO^-$ treatment from Amadori product, we incubated glycated HSA or nonglycated HSA (2 mg/ml) with $ONOO^-$ (6 mmol/l) at 37°C for 5 min, followed by determination of CML by noncompetitive ELISA. As shown in Fig. 1*A*, a significant amount of CML was generated in the presence of glycated HSA, whereas CML was at background levels in the experiment with nonglycated HSA. To confirm the interaction of $ONOO^-$ with these proteins, we also determined the amount of nitrotyrosine, a $ONOO^-$-modified tyrosine, in glycated and nonglycated HSA. As shown in Fig. 1*B*, similar amounts of nitrotyrosine were detected in both glycated and nonglycated HSA, suggesting that protein modification by $ONOO^-$ was independent of the presence of the Amadori product.

Detection of CML and nitrotyrosine by HPLC. To confirm the results obtained from ELISA, we measured CML and nitrotyrosine levels in glycated HSA incubated with 6 mmol/l $ONOO^-$ (Fig. 1) by amino acid analysis. Compared with glycated HSA not exposed to $ONOO^-$ that contained <0.01 mol CML/mol glycated HSA (Fig. 1*A*), there was 1.2 mol of CML/mol glycated HSA treated with $ONOO^-$ (Fig. 1*B*), indicating that 18% of fructoselysine (1.2 of 6.5 fructoselysine) in glycated HSA was converted to CML. Furthermore, 1.5 mol of nitrotyrosine/mol of glycated HSA treated with $ONOO^-$ was measured, representing modification of 8% of tyrosine residues (1.5 of 18

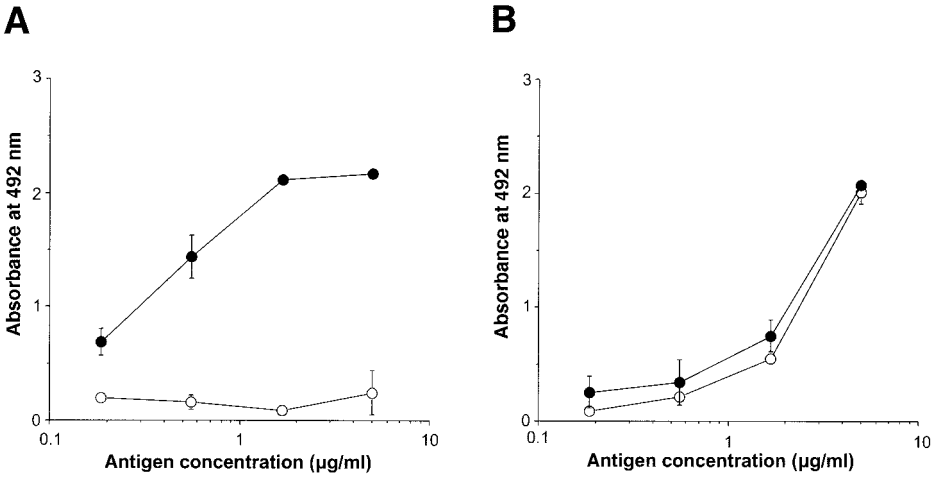

FIG. 1. Effect of $ONOO^-$ on the formation of CML and nitrotyrosine. Glycated HSA or nonglycated HSA (2 mg/ml) was incubated with $ONOO^-$ (6 mmol/l) at 37°C for 5 min, and amounts of CML (*A*) and nitrotyrosine (*B*) content were determined by noncompetitive ELISA. Each well of a 96-well microtiter plate was coated with $ONOO^-$-treated glycated HSA (●) or nonglycated HSA (○), incubated for 1 h, and then assayed using 6D12 (*A*) or 2H1 (*B*) antibodies. Antibodies bound to wells were detected using HRP-conjugated anti-mouse IgG antibody as described in RESEARCH DESIGN AND METHODS. Data are mean ± SD (n = 3).

tyrosine). CML and nitrotyrosine were not detected in glycated HSA not exposed to $ONOO^-$ (Fig. 2*A*), indicating that CML and nitrotyrosine in glycated HSA were generated by exposure to $ONOO^-$.

Dose-dependent effects of $ONOO^-$ on CML formation from Amadori product. For elucidating the effect of $ONOO^-$ on CML formation from the Amadori product, glycated HSA was incubated at 37°C for 1 h in the presence of different concentrations of $ONOO^-$, followed by determination of CML content by ELISA. As shown in Fig. 3, CML increased in a dose-dependent manner.

Effect of preincubation of $ONOO^-$ with glucose on CML formation. Because CML is also generated by protein modification with the reactive α-oxoaldehyde glyoxal, which can be generated by oxidative cleavage of glucose (26), we also determined the effect of preincubation of $ONOO^-$ with glucose on CML formation. As shown in Fig. 4, CML formation from $ONOO^-$-treated glucose increased with increasing concentrations of $ONOO^-$. These results suggested that certain aldehydes, such as glyoxal, generated during the incubation of glucose with $ONOO^-$, promoted CML formation.

Effect of aminoguanidine on CML formation. α-Oxoaldehydes, including glyoxal, 3-deoxyglucosone, and methylglyoxal, are known to contribute to AGE formation (21). We determined the effect of the carbonyl trap (27) aminoguanidine on CML formation from both the Amadori product and $ONOO^-$-treated glucose; aminoguanidine reacts with α-oxoaldehydes to form aminotriazine derivatives (28). First, glycated HSA was incubated with $ONOO^-$

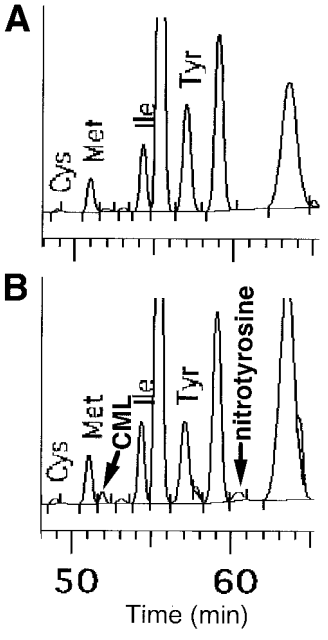

FIG. 2. Detection of CML and nitrotyrosine by HPLC analysis. Glycated HSA was incubated with 6 mmol/l $ONOO^-$ at 37°C for 5 min and then hydrolyzed with 6 N of HCl for 24 h at 110°C as described in RESEARCH DESIGN AND METHODS. Chromatograms are glycated HSA (*A*) and $ONOO^-$-treated glycated HSA (*B*). Elution time for CML was 51.9 min, and that for nitrotyrosine was 60.4 min.

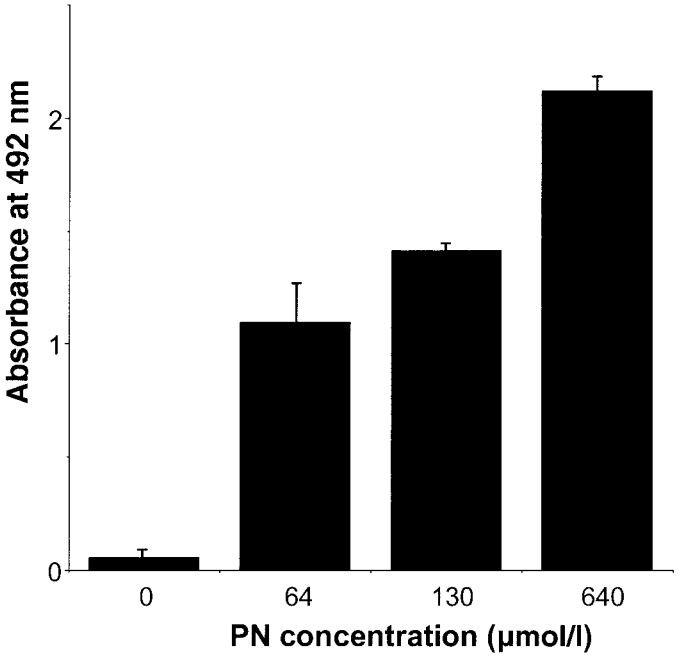

FIG. 3. Dose-dependent effects of $ONOO^-$ on the formation of CML. Glycated HSA was incubated at 37°C for 1 h with the indicated concentrations of $ONOO^-$ (64–640 μmol/l), followed by determination of CML by noncompetitive ELISA. $ONOO^-$-treated samples (5 μg/ml) were coated on the ELISA plate and incubated for 1 h. The wells were washed and blocked with gelatin, followed by reaction for 1 h with 0.1 μg/ml 6D12. The antibodies bound to wells were detected by HRP-conjugated anti-mouse IgG antibody. A total of 0.72 mol of CML/mol of glycated HSA was measured by HPLC in 640 μmol/l $ONOO^-$-treated glycated HSA. Data are mean ± SD (n = 3).

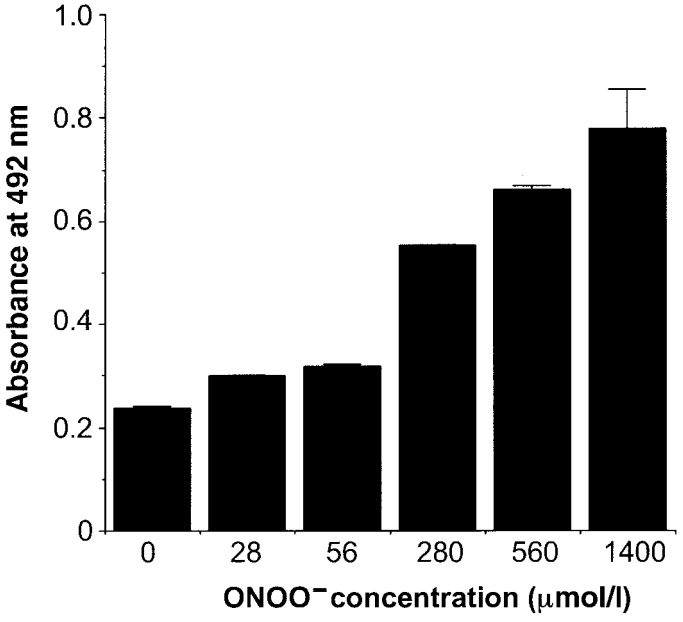

FIG. 4. Effect of $ONOO^-$ on CML formation from $ONOO^-$-treated glucose. D-Glucose (36 mmol/l) was incubated at 37°C for 30 min at the indicated concentrations of $ONOO^-$ and further incubated at 37°C for 24 h in the presence of HSA (1.4 mg/ml), followed by determination of CML content by ELISA. The immunoreactivity of 6D12 with a fixed concentration of antigen (5 μg/ml) was determined by absorbance at 492 nm. A total of 0.38 mol of CML/mol of HSA was measured by HPLC in 1,400 μmol/l $ONOO^-$-treated glucose. Data are mean ± SD (n = 3).

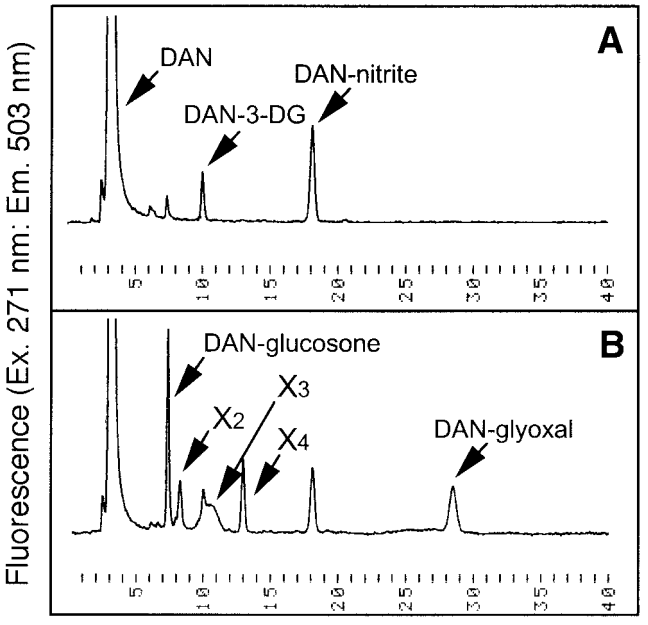

FIG. 6. Detection of α-oxoaldehydes. *A*: Degraded $ONOO^-$ solution was incubated with 142 mmol/l glucose at 37°C for 5 min and further incubated in the presence of 0.47 mmol/l DAN. DAN–α-oxoaldehyde adducts were analyzed by HPLC equipped with fluorescence detector (λ ex/em = 271/503 nm) as described in RESEARCH DESIGN AND METHODS. *B*: DAN adducts formed when glucose (142 mmol/l) was incubated at 37°C for 5 min with 2.5 mmol/l $ONOO^-$ and further incubated in the presence of 0.47 mmol/l DAN. Five new peaks were detected at 7.4, 8.3, 10.5, 13.0, and 28.5 min, respectively. The peak at retention time of 28.5 min was confirmed as DAN-glyoxal adduct by comparison with authentic standard. ESI mass spectroscopic analysis of the peak eluted at retention time of 7.4 min indicated that it was a DAN-glucosone adduct. The structures of X_2–X_4 are under investigation. Concentrations of glucosone and glyoxal in $ONOO^-$-treated glucose were 11 and 8.1 μmol/l, respectively.

in the presence of aminoguanidine. As shown in Fig. 5*A*, aminoguanidine did not inhibit CML formation from glycated HSA, indicating that CML was generated directly from the Amadori product. Next, glucose was preincubated with $ONOO^-$, followed by further incubation with HSA in the presence of aminoguanidine. As shown in Fig. 5*B*, CML formation from $ONOO^-$-treated glucose was significantly (>70%) inhibited by 9 mmol/l aminoguanidine and completely inhibited at 90 mmol/l aminoguanidine, arguing that α-oxoaldehydes contributed to CML formation from $ONOO^-$-treated glucose.

Detection of α-oxoaldehydes by DAN. Because CML formation from $ONOO^-$-treated glucose pathway was totally inhibited by aminoguanidine, we sought to identify the α-oxoaldehyde(s) generated from glucose during exposure to $ONOO^-$. First, we determined the contribution of nitrite, degradation product of $ONOO^-$, to the formation of α-oxoaldehydes. $ONOO^-$ solution was incubated at 37°C for 24 h to be degraded into nitrite. After confirming the elimination of $ONOO^-$ by absorbance at 302 nm, nitrite solution was incubated with glucose, followed by determination of α-oxoaldehydes by DAN. As shown in Fig. 6*A*, three separated peaks emerged at 3.0, 10.0, and 18.1 min. By comparison with authentic standards, the peak at 3.0

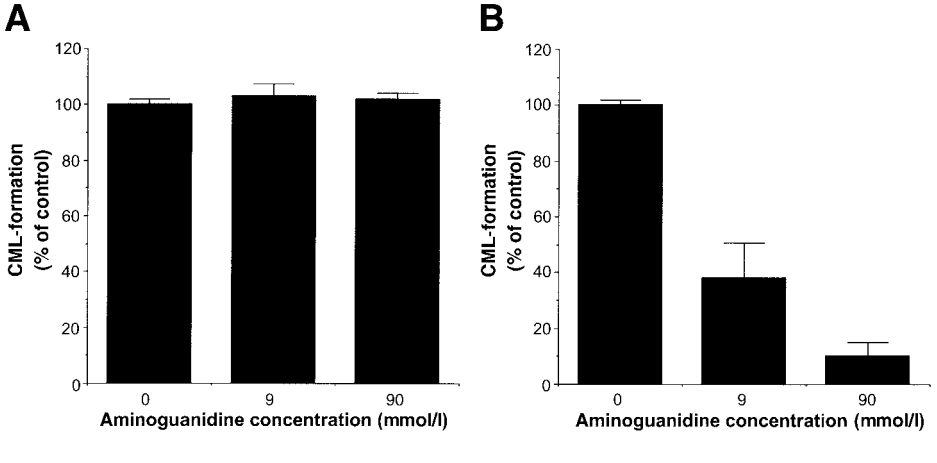

FIG. 5. Effect of aminoguanidine on CML formation. *A*: Glycated HSA (2 mg/ml) was incubated at 37°C for 1 h with 2.7 mmol/l $ONOO^-$ in the presence of indicated concentrations of aminoguanidine, and immunoreactivity of 6D12 at a fixed concentration (5 μg/ml) of antigen was determined by absorbance at 492 nm. Data are expressed as percentage of NO aminoguanidine control. A total of 0.91 mol of CML/mol of glycated HSA in NO aminoguanidine sample was measured by HPLC. Absorbance at 492 nm of NO aminoguanidine sample was 1.244. *B*: D-Glucose (36 mmol/l) was incubated at 37°C for 20 min with 560 μmol/l $ONOO^-$ and further incubated with HSA in the presence of indicated concentrations of aminoguanidine, followed by determination of CML content by noncompetitive ELISA. Data are expressed as percentage of NO aminoguanidine control. A total of 0.29 mol of CML/mol of HSA in NO aminoguanidine sample was determined by HPLC. Absorbance at 492 nm of NO aminoguanidine sample was 0.734. Data are mean ± SD (n = 3).

min was identified as free DAN, the peak at 10.0 min was identified as DAN-3-deoxyglucosone adduct, and the peak at 18.1 min was identified as the DAN-nitrite adduct (29).

Next, we determined the effect of $ONOO^-$ on the formation of α-oxoaldehydes. When glucose was incubated with $ONOO^-$ and then further incubated with DAN, five new peaks were detected at 7.4, 8.3, 10.5, 13.0, and 28.5 min (Fig. 6*B*), respectively. The peak at retention time of 28.5 min was confirmed as glyoxal by comparison with an authentic standard. The main peak, eluting at retention time of 7.4 min, was isolated, followed by determination of its molecular weight. ESI-MS spectroscopic analysis revealed an ion with m/z 300, suggesting the formation of DAN-glucosone ($N_2C_{16}H_{16}O_4$; data not shown). Furthermore, the authentic DAN-glucosone adduct yielded a peak with the same retention time present in the sample, indicating that the peak eluting at 10.0 min was a DAN-glucosone adduct. The concentrations of glucosone and glyoxal in $ONOO^-$-treated glucose were 11 and 8.1 μmol/l for glucosone and glyoxal, respectively. When 200 mmol/l glucosone was incubated at 37°C for 1 week with BSA (2 mg/ml) in 200 mmol/l phosphate buffer (pH 7.4), 6.4 mol of CML/mol of BSA was detected by amino acid analysis, indicating that glucosone contributes to the formation of CML.

DISCUSSION

Recent studies have demonstrated accumulation of CML, a major AGE structure, in numerous tissues in diabetes and atherosclerosis. CML is known to be derived from the Amadori product by oxidative cleavage (23) but also from glyoxal formed on autoxidation of glucose. The specific question addressed in the present study was whether CML formation is enhanced by $ONOO^-$, which is generated from the reaction of NO with O_2^-.

CML was readily formed on treatment of glycated HSA with $ONOO^-$ (Figs. 1 and 2) and also on exposure of protein to $ONOO^-$-treated glucose (Fig. 4). In both cases, the yield of CML increased with increasing concentration of $ONOO^-$ (Figs. 3 and 4). CML formation from $ONOO^-$-treated glucose but not from Amadori-modified protein was inhibited by aminoguanidine (Fig. 5*B*), a trapping reagent for α-oxoaldehydes. Incubation of glucose with $ONOO^-$ (Fig. 6) resulted in the production of α-oxoaldehydes, including glyoxal, glucosone, and several unidentified dicarbonyl compounds (Fig. 6). However, because glucosone degrades rapidly in the presence of transition metal (30), CML might be generated from the reaction of BSA with glucosone or its degradation products during the incubation of BSA with glucosone. Considered together, our results indicate that glucosone and glyoxal, both generated from $ONOO^-$-treated glucose, reacted with protein to form CML (Fig. 7). When glucose was incubated without $ONOO^-$, 3-deoxyglucosone was also observed (Fig. 6*A*). Because 3-deoxyglucosone content in the sample is independent of $ONOO^-$ (Fig. 6*A* and *B*), generation of 3-deoxyglucosone might not be derived from interaction of $ONOO^-$ with glucose but by time-dependent degradation of glucose (31). In addition to known α-oxoaldehydes, we detected several new DAN adducts. Because analogues of 3-deoxyglucosone such as 1-deoxyglucosone and 4-deoxyglucosone are known to be generated during the autoxidation of glucose (28), X_2, X_3, and X_4 may be some of these deoxyglucosone analogues. Furthermore, because aminoguanidine did not show any inhibitory effect on CML formation from glycated HSA (Fig. 5*B*), CML is likely to be generated directly by the cleavage of Amadori product through interaction with $ONOO^-$ (Fig. 7).

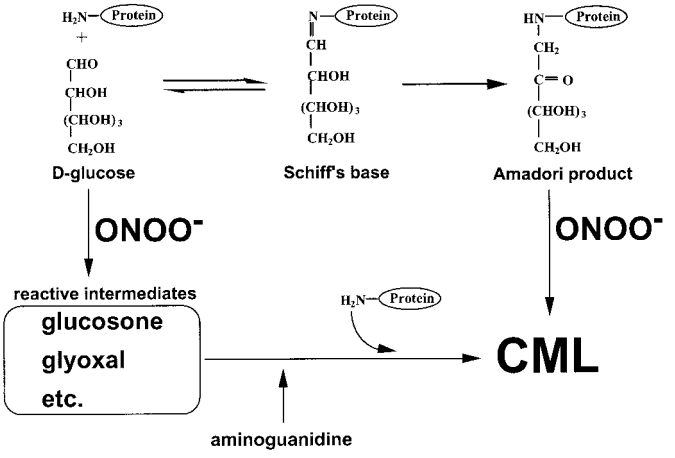

FIG. 7. Proposed pathway for $ONOO^-$-induced CML formation.

In summary, there are two major findings in the present study. The first is that $ONOO^-$ is involved in CML formation from glycated HSA by oxidative cleavage of the Amadori product. Second, glucosone and glyoxal were generated from glucose by $ONOO^-$ treatment and contributed to further CML formation. The reaction of protein with $ONOO^-$ is known to induce oxidation of amino acids such as tryptophan, cysteine, and tyrosine (16). Our results demonstrate a novel pathway for $ONOO^-$-induced protein modification by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose. Therefore, our results suggest that a proportion of glyoxal detected in vivo might be generated by $ONOO^-$-mediated oxidation of glucose and contribute to CML formation.

Production of NO is known to increase in diabetic conditions. Stevens et al. (32) demonstrated that streptozotocin-induced diabetic rats and mice exhibit high NO production and expression of mRNA for inducible NO synthase (iNOS) in macrophages and that these levels decreased on insulin administration. They speculated that transforming growth factor-β1 (TGF-β1) may contribute to insulin-mediated regulation of NO production because *1*) macrophages of diabetic mice produce less TGF-β1 than normal mice, *2*) the circulating levels of TGF-β1 are lower in diabetic mice than in normal mice, and *3*) insulin administration increases circulating TGF-β1 in normal mice (32). Furthermore, production of O_2^-, which reacts with NO to produce $ONOO^-$, also increases in hyperglycemia (33). Sakurai and Tsuchiya (34) demonstrated that O_2^- was generated from the Amadori product and was converted into hydrogen peroxide by dismutation (35). Moreover, incubation of porcine aorta endothelial cells in the presence of glycated LDL is known to generate O_2^- and reduce NO stability (19). Considered together, these reports strongly suggest that formation of $ONOO^-$ increases in diabetes-associated pathological processes. $ONOO^-$ is responsible for nitration of tyrosine residues in proteins; therefore, the presence of nitrotyrosine in

plasma proteins is considered indirect evidence of $ONOO^-$ production. Actually, nitrotyrosine was found in the plasma of all patients with type 2 diabetes (0.251 and 0.141 mmol/l), whereas it was not detected in the plasma of healthy control subjects (36), indicating high $ONOO^-$ production in patients with diabetes.

Although the lifetime of hydroxyl radical is 1×10^{-9} s, the physiological half-life of $ONOO^-$, estimated at 1.9 s (15), is sufficiently long to allow $ONOO^-$ to diffuse distances equal to cellular diameters (37). $ONOO^-$ also directly oxidizes sulfhydryl groups at a 1,000-fold greater rate than hydrogen peroxide (38) and initiates lipid peroxidation without requirement of a transition metal (39). Thus, $ONOO^-$ is highly reactive and potentially a major oxidant and source of CML in vivo even in the absence of free metal ions. The concentration of CO_2 in vivo is 1 mmol/l, and the rate constant for reaction of CO_2 with $ONOO^-$ is $5.8 \times 10^4\ mol \cdot l^{-1} \cdot s^{-1}$ (37). To demonstrate the physiological conditions, we determined $ONOO^-$-induced CML formation in the presence of 20 mmol/l sodium hydrogen carbonate as described in RESEARCH DESIGN AND METHODS. Sodium hydrogen carbonate did not show any inhibitory effect on $ONOO^-$-induced CML formation from both glycated HSA and $ONOO^-$-treated glucose pathways (data not shown).

Ischiropoulos et al. (40) demonstrated that the majority of NO produced by activated rat alveolar macrophages was converted to $ONOO^-$ (estimated at $0.11\ nmol \cdot 10^6\ cells^{-1} \cdot min^{-1}$). They concluded that if all macrophages in a lung were activated, then the average rate of $ONOO^-$ formation would be $0.8\ mmol \cdot l^{-1} \cdot min^{-1}$ within the whole lung and $1\ mmol \cdot l^{-1} \cdot min^{-1}$ in the epithelial lining fluid (40). Although actual concentrations of $ONOO^-$ in vivo have not yet been measured, $ONOO^-$ concentration used in the present study might reflect pathological conditions rather than normal conditions.

We previously demonstrated that the hydroxyl radical, which was generated by the Fenton reaction between Fe^{2+} and Amadori-derived hydrogen peroxide, plays an important role in the formation of CML from glycated HSA (23). Because the rate constant of $ONOO^-$ formation ($7 \times 10^9\ mol \cdot l^{-1} \cdot s^{-1}$) is 1,000-fold higher than that of the hydroxyl radical (41) and, as described above, the half-life of $ONOO^-$ is 10^9 times longer than that of hydroxyl radical under physiological conditions (15), it is highly likely that the formation of CML in vivo is mediated by $ONOO^-$ rather than by hydroxyl radical.

In addition to the presence of nitrotyrosine in the plasma of patients with type 2 diabetes, nitrotyrosine has been detected in atherosclerotic lesions of formalin-fixed human coronary arteries (41). As described above, because CML is localized in human atherosclerosis lesions (7–9), a portion of the CML detected in atherosclerosis lesions would be generated by $ONOO^-$.

Finally, Sugimoto et al. (42) demonstrated that both CML and iNOS were detected in the mesangial area in glomeruli of streptozotocin-induced diabetic rats at 52 weeks, and both decreased after treatment with aminoguanidine, an inhibitor for AGE formation. They concluded that CML possibly enhances the expression of iNOS by stimulating the expression of tumor necrosis factor-α in diabetic glomeruli (42). In conclusion, our study indicates that $ONOO^-$-mediated CML formation may play an important role in the formation of AGEs and in the pathogenesis of diabetic complications.

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