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Original Research Communication

Serum 4-Hydroxy-2-Nonenal-Modified Albumin Is Elevated in Patients with Type 2 Diabetes Mellitus

SHINYA TOYOKUNI,[1] SATOSHI YAMADA,[2] MINORU KASHIMA,[2] YU IHARA,[3] YUICHIRO YAMADA,[3] TOMOYUKI TANAKA,[1] HIROSHI HIAI,[1] YUTAKA SEINO,[3] and KOJI UCHIDA[4]

ABSTRACT

4-Hydroxy-2-nonenal (HNE) is one of the major lipid peroxidation products with cytotoxic and mutagenic activity. It further reacts with protein residues such as histidine to generate stable Michael adducts. To evaluate the status of oxidative stress in the serum of type 2 diabetes mellitus, we constructed a sandwich enzyme-linked immunosorbent assay to measure serum HNE-modified albumin by the use of a specific monoclonal antibody (HNEJ-2) against HNE-histidine adducts as well as an antibody against human serum albumin. Serum of type 2 diabetes outpatients revealed significantly higher levels of HNE-modified albumin (736.1 ± 34.2 pmol/ml, $n = 54$) than the matched nondiabetics (611.4 ± 39.1 pmol/ml, $n = 30$; means ± SEM; $p = 0.018$). However, no significant correlation was observed in diabetic outpatients between the levels of HNE-modified albumin and clinical parameters such as fasted blood glucose, HbA1c, diabetes duration, or complications. Our data demonstrated the increased formation of serum HNE-modified albumin in type 2 diabetic outpatients in the milieu between liver and vascular lumina, indicating the presence of oxidative stress. Antiox. Redox Signal. 2, 681–685.

INTRODUCTION

OVERLOADS OF REACTIVE OXYGEN SPECIES (ROS) that exceed the capacity of antioxidant system induce oxidative stress in cells. Oxidative stress has been associated with a number of pathological phenomena including inflammation, carcinogenesis, aging, atherosclerosis, and reperfusion injury. Oxidative stress may play a role in the pathophysiology of diabetes mellitus because prolonged exposure to hyperglycemia induces nonenzymatic glycation of proteins through Maillard's reaction. The resulting products, such as Schiff base and Amadori products, can lead to the production of ROS (Sakurai and Tsuchiya, 1988; Njoroge and Monnier, 1989; Baynes, 1991).

We and other investigators have shown that the level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidatively modified DNA adduct, is increased either in the urine (Leinonen *et al.*, 1997) or in the mononuclear cells (Dandona *et al.*, 1996) of type 2 diabetic patients. Furthermore, we have found that hyperglycemia causes oxidative stress in the pancreatic β cells of GK rats, a model of type 2 diabetes (Ihara *et al.*, 1999). Recently, increased autoantibody titers against malondialdehyde- or 4-hydroxy-2-nonenal (HNE)-modified albumin in diabetic rats were reported (Traverso *et al.*, 1998). However, thus far, there is no demonstration of

[1]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[2]Tsukuba Laboratory, Japan Oil Company, Tsukuba, Japan.
[3]Department of Metabolism and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[4]Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagriculture, Nagoya, Japan.

serum HNE-modified albumin as a possible epitope for the autoantibodies in type 2 diabetic patients. In the present study, we have focused on the demonstration of serum HNE-modified albumin by constructing a sandwich enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody against HNE-histidine adducts (Toyokuni *et al.*, 1995).

MATERIALS AND METHODS

Chemicals

HNE was from Cayman Chemical (Ann Arbor, MI) and human serum albumin (HSA) was from Sigma Chemical Co. (St. Louis, MO). Tween 20, *o*-phenylenediamine, and hydrogen peroxide were from Wako (Osaka, Japan). All the chemicals used were of analytical quality. Blockace was from Dainihon-Seiyaku (Osaka, Japan). ELISA microtiter plates (F16 MaxiSorp) were from Nalge Nunc International (Tokyo, Japan). Protein concentration was determined with BCA reagent (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard.

Antibodies

A mouse monoclonal antibody against HNE-histidine adducts (HNEJ-2) developed in our laboratory (Toyokuni *et al.*, 1995) and a peroxidase-conjugated antibody against HSA (The Binding Site Ltd., Birmingham, England) were used.

HNE-HSA standard

HSA (5 mg/ml) in 50 m*M* phosphate-buffered saline (PBS) pH 7.2, were incubated at 37°C for 2 hr in the presence of 1 m*M* HNE. The product was extensively dialyzed in 50 m*M* PBS, and was used as an HNE-HSA standard.

Experimental procedures for ELISA

Microtiter plate wells were precoated with 100 μl of 20 μg/ml monoclonal antibody HNEJ-2 for 16 hr at 4°C, washed with Dulbecco's PBS four times, then blocked with 200 μl of diluted blockace (vol/vol = 1:4 with H_2O) for 2 hr at room temperature. After discarding the blocking solution, appropriately diluted serum sample with blockace (100 μl) was added and incubated for 2 hr at room temperature, and then washed with Dulbecco's PBS four times. Thereafter, 100 μl of peroxidase-conjugated antibody against HSA (diluted to 1:2,500 to 5,000 with 0.05% Tween/PBS) was added and incubated for 2 hr at room temperature. After washing with 0.05% Tween/PBS four times, 0.125% (wt/vol) *o*-phenylenediamine in 50 m*M* citrate- and phosphate-buffer, pH 5.0, in the presence of 0.02% (vol/vol) H_2O_2 was added for color presentation. The reaction period was 10–20 min at 25°C, followed by addition of 50 μl of 2 *N* sulfuric acid to terminate the reaction. The plate was measured at 492 nm with a microplate reader (M-EMax, Molecular Devices, Menlo Park, CA). Measurements were

TABLE 1. BIOCHEMICAL, CLINICAL, AND DEMOGRAPHIC CHARACTERISTICS OF THE STUDY GROUPS

	Type 2 diabetic patients	*Control subjects*
Gender (male/female, *n*)	23/31	11/19
Age (years)	60.3 ± 13.8	64.4 ± 9.0
Smokers (*n*)	2 (3.7%)	2 (6.7%)
Duration of disease (years)	11.8 ± 7.0	—
BMI (kg/m^2)	23.0 ± 3.2	22.9 ± 2.8
Fasting blood glucose (mg/dl)	165.7 ± 37.8**	95.0 ± 7.7
HbA1c (%)	8.1 ± 1.2	—
Total cholesterol (mg/dl)	195.8 ± 29.7	203.0 ± 34.3
Serum albumin (g/dl)	3.8 ± 0.4*	4.1 ± 0.4
Blood pressure (mmHg, systolic/diastolic)	133 ± 19/74 ± 9.4	123 ± 11/72 ± 6.9
Neuropathy (+/−)	23/31	—
Nephropathy (+/−)	34/20	—
Retinopathy (+/−)	24/30	—

Data are means ± SD. *$p < 0.005$; **$p < 0.001$.

done in duplicate, and the mean was used as the data.

Serum samples

The study group consisted of 54 type 2 diabetes mellitus outpatients at two affiliated hospitals of Kyoto University. The patients fulfilled the WHO diagnostic criteria for type 2 diabetes mellitus. Thirty nondiabetic attendants of the same hospitals, matched for age and gender, were used as control subjects. All subjects gave written informed consent. Biochemical, clinical, and demographic characteristics of the study groups are presented in Table 1. At the time of study, 8 patients were treated with diet alone, 32 patients with one or more antidiabetic drugs, 13 patients with insulin, and 1 patient both with insulin and a diabetic drug. Blood samples were obtained in the morning before breakfast.

Statistical analyses

Statistical calculation included determination of mean ± SEM or SD, Student's *t*-test, and evaluation of linear regression. Welch's correction, which does not assume equal variances, was applied when necessary.

RESULTS

The sandwich ELISA system revealed a linear response in the range up to 200 pmol HNE-modified HSA per well (Fig. 1). Therefore, this range was used for the sample measurements. The type 2 diabetes group and the control group were matched for age and gender. There was no significant difference in the ratio of smokers, body mass index (BMI), total cholesterol, and blood pressure between the two groups; the diabetes group showed significantly higher fasted blood glucose and significantly lower serum albumin levels (Table 1).

Serum HNE-modified albumin was significantly higher in the diabetes group (736.1 ± 34.2 pmol/ml) than the control group (611.4 ± 39.1 pmol/ml, means ± SEM; $p = 0.018$). Because the diabetes group showed lower serum albumin levels, the difference in serum HNE-modified albumin/serum albumin level was more significant (17.59 ± 0.84 vs. 14.54 ± 0.95 nmol/g serum albumin). This implies that 0.114% of serum albumin is modified by HNE in diabetic patients whereas 0.0945% is modified in the control subjects. There was a proportionate association between the levels of serum HNE-modified albumin and those divided by each serum albumin level ($r = 0.961$, $p < 0.0001$).

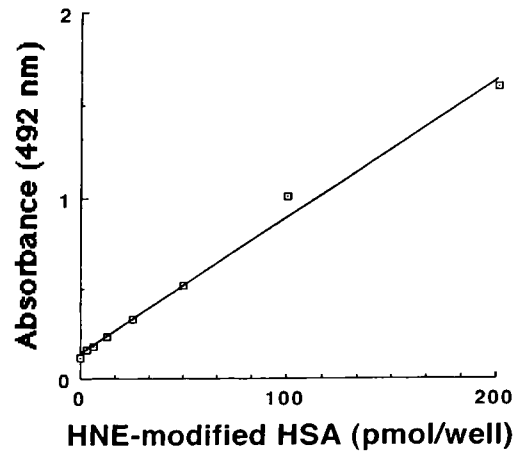

FIG. 1. Standard curve of sandwich ELISA system for HNE-modified human serum albumin. See Materials and Methods for experimental detail.

Furthermore, linear regression was evaluated between the levels of HNE-modified serum albumin and the presence of diabetic complications (retinopathy, neuropathy, and nephropathy) in addition to each factor shown in Table 1. However, no significant correlation was observed between the levels of HNE-modified serum albumin and each factor studied.

DISCUSSION

HNE is an α,β-unsaturated aldehyde that can be formed by peroxidation of ω_6-unsaturated fatty acids. In the human body, it originates almost exclusively from phospholipid-bound arachidonic acid and may be the most reliable index of ROS-induced lipid peroxidation. HNE exhibits a variety of cytopathological effects such as enzyme inhibition, inhibition of DNA and RNA synthesis, inhibition of protein synthesis, and induction of heat shock proteins. It is highly cytotoxic to many types of cells such as hepatocytes, fibroblasts, and Ehrlich ascites

tumor cells. HNE further shows mutagenic and genotoxic effects as well as inhibitory effects on cellular proliferation (Esterbauer *et al.*, 1991; Parola *et al.*, 1999). In a cell culture system using Ehrlich ascites tumor cells, more than 80% of HNE is metabolized to nontoxic compounds, either by oxidation or reduction, within 10 min after exposure (Grune *et al.*, 1994). It has been proposed that HNE exerts these effects by its facile reactivity with biological molecules, particularly with proteins. Recently, we found that HNE impairs by covalent modification proteasome activity which lyses intracellular HNE-modified proteins, leading to further accumulation of the modified proteins via vicious cycle (Okada *et al.*, 1999).

Serum albumin is synthesized by adult liver (Dugaiczyk *et al.*, 1982) and belongs to a multigene family of proteins that contribute to colloid osmotic blood pressure and aid in the transport, distribution, and metabolism of many endogenous and exogenous ligands (Pardridge, 1987). In the present study, we demonstrated that serum HNE-modified albumin does exist in nondiabetic control subjects as one modified form in 1,058 molecules on average and as one in 877 molecules in type 2 diabetic patients. This implies that oxidative stress is imposed in the serum environment of diabetic patients. Higher levels of serum HNE-modified albumin might work as an antigen and contribute to the autoantibody production, as was shown in the experiments of diabetic rats (Traverso *et al.*, 1998).

The levels of serum HNE-modified albumin were analyzed against the indicated factors. However, more of them showed significant correlation, including diabetes duration and its complications, whereas only the diabetic condition as a whole was associated. More detailed studies using serum samples from inpatients are now in progress.

It is not known so far where the protein modification of this kind takes place—whether in the hepatocytes before secretion or in the vascular lumina. Recently, it was reported based on an *in vitro* study that lipoperoxidative aldehydes accumulate in liver microsomes and mitochondria at a higher rate in spontaneously diabetic BB/WOR rats than in control nondiabetic animals (Traverso *et al.*, 1999). While it is generally thought that half-life of modified proteins, particularly oxidized proteins, are shorter (Davies and Goldberg, 1987), how serum HNE-modified albumin is metabolized is not known. In the next study, it is necessary to elucidate the metabolic pathways of serum HNE-modified albumin and also to evaluate which serum protein is most vulnerable to this type of modification in the diabetic condition. We believe that this work will be helpful for the assessment of oxidative stress in diabetic patients and further warrants the trial of supplementary antioxidant therapies for type 2 diabetes mellitus.

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ABBREVIATIONS

BMI, Body mass index; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HNE, 4-hydroxy-2-nonenal; HSA, human serum albumin; 8-OHdG 8-hydroxy-2′-deoxyguanosine; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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Address reprint requests to:
Dr. Shinya Toyokuni
Department of Pathology and Biology of Diseases
Graduate School of Medicine
Kyoto University
Kyoto 606-8501, Japan

E-mail: toyokuni@path1.med.kyoto-u.ac.jp

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