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REVIEW ARTICLE

Lipid glycation and protein glycation in diabetes and atherosclerosis

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Abstract Recent instrumental analyses using a hybrid quadrupole/linear ion trap spectrometer in LC-MS/MS have demonstrated that the Maillard reaction progresses not only on proteins but also on amino residues of membrane lipids such as phosphatidylethanolamine (PE), thus forming Amadori-PE (deoxy-D-fructosyl PE) as the principal products. The plasma Amadori-PE level is 0.08 mol% of the total PE in healthy subjects and 0.15–0.29 mol% in diabetic patients. Pyridoxal 5′-phosphate and pyridoxal are the most effective lipid glycation inhibitors, and the PE-pyridoxal 5′-phosphate adduct is detectable in human red blood cells. These findings are beneficial for developing a potential clinical marker for glycemic control as well as potential compounds to prevent the pathogenesis of diabetic complications and atherosclerosis. Glucose and other aldehydes, such as glyoxal, methylglyoxal, and glycolaldehyde, react with the amino residues of proteins to form Amadori products and Heynes rearrangement products. Because several advanced glycation end-product (AGE) inhibitors such as pyridoxamine and benfotiamine inhibit the development of retinopathy and neuropathy in streptozotocin (STZ)-induced diabetic rats, AGEs may play a role in the development of diabetic complications. In the present review, we describe the recent progress and future applications of the Maillard reaction research regarding lipid and protein modifications in diabetes and atherosclerosis.

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Introduction

Oxidative stress on lipid molecules plays a role in the pathophysiology of atherogenesis, diabetes, aging, and other conditions (Witztum and Steinberg 1991). To determine lipid hydroperoxides as a primary oxidation product, Miyazawa (Miyazawa 1989; Miyazawa et al. 1992) established the chemiluminescence detection-liquid chromatography (CL-HPLC) method. Using this method, the plasma phosphatidylcholine hydroperoxide (PCOOH) concentration was confirmed to increase in hyperlipidemic patients (Kinoshita et al. 2000) and in type 2 diabetic patients (Nagashima et al. 2002). Therefore, it was hypothesized that plasma PCOOH formation is closely involved in the pathophysiology of these diseases. To understand the increase in PCOOH in diabetic plasma, "lipid glycation" has been studied in biomembranes (Lertsiri et al. 1998).

Lipid glycation refers to the reaction between aminophospholipids such as phosphatidylethanolamine (PE) and glucose during the Maillard reaction. The reaction involves Schiff base formation and rearrangement to a PE-linked Amadori product (Amadori-PE) (Fig. 1) (Lertsiri et al. 1998) (Bucala et al. 1993). In vitro studies on the Maillard reaction have demonstrated that Amadori product autoxidation may lead to the formation of reactive oxygen species. The reactive oxygen species are likely to cause peroxidation of unsaturated fatty acid residues in membrane lipids, which propagate free radical reactions and lead to PCOOH formation. Because the Maillard reaction, occasionally referred to as a glycation, occurs widely in various biological systems, the reaction may play an important and primary role in initiating lipid

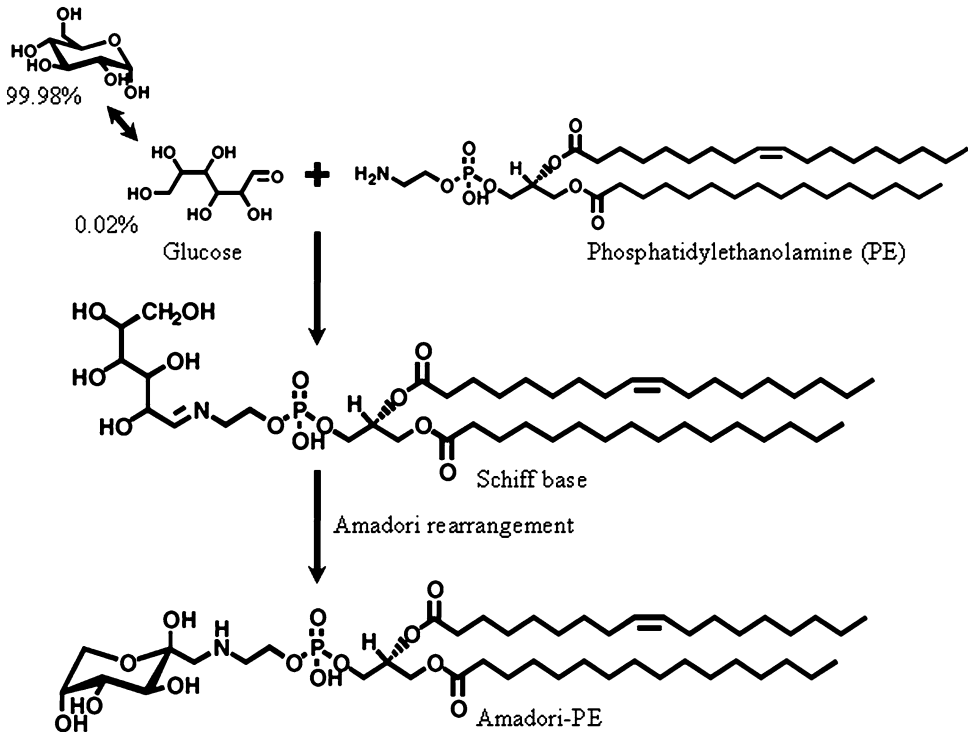

Fig. 1 Scheme for the glycation of phosphatidylethanolamine (PE). Glucose reacts with the amino group of PE to form an unstable Schiff base, which undergoes an Amadori rearrangement to yield the stable PE-linked Amadori product (Amadori-PE; deoxy-D-fructosyl phosphatidylethanolamine)

peroxidation in vivo. Lipid peroxidation is involved in the pathophysiology of atherogenesis, diabetes, and aging.

Advanced glycation end-products (AGEs) are formed via nonenzymatic glycation of proteins with reducing sugars, such as glucose, glucose 6-phosphate, fructose, ribose and intermediate aldehydes, and are composed of heterogeneous structures characterized by a yellow–brown color, autofluorescence, and intra- and intermolecular cross-linking. Aldehydes react with cationic amino residues on amino acids such as lysine and arginine and increase those molecular masses and negative charges (Fig. 2) (Nagai et al. 2000) (Mera et al. 2010). The AGE research originally began in the field of food chemistry and has been enormously expanded in vivo since the 1990s because of the development of polyclonal and monoclonal antibodies against AGE-modified proteins. For instance, N^{ε}-(Carboxymethyl)lysine (CML), a major antigenic AGE structure, is involved in normal aging (Araki et al. 1992) as well as in the pathogenesis of several age-enhanced diseases, such as diabetic nephropathy (Imai et al. 1997), atherosclerosis (Kume et al. 1995), diabetic retinopathy (Hammes et al. 1996), hemodialysis-associated amyloidosis (Miyata et al. 1993), chronic renal failure (Yamada et al. 1994), and Alzheimer's disease (Smith et al. 1994). CML is generated by the oxidative cleavage of Amadori products by the hydroxyl radical (Nagai et al. 1997), peroxynitrite (Nagai et al. 2002) and hypochlorous acid (Fig. 3) (Mera et al. 2007), therefore suggesting that CML is an important biological marker of oxidative stress in vivo.

Pentosidine is an AGE structure which possesses fluorescent and cross-linking properties (Sell and Monnier 1989) and can be a glycoxidation marker for AGEs in proteins (Baynes 1991). Furthermore, a dramatic increase in pentosidine levels is observed in the plasma of patients with end-stage renal failure (Odetti et al. 1992) (Taneda and Monnier 1994), β2-microglobulin amyloid deposits (Miyata et al. 1996), and skin collagens (Hricik et al. 1993) irrespective of the presence or absence of diabetes. Pentosidine has therefore been implicated in tissue damage not only in diabetic patients but also in hemodialysis patients with end-stage renal failure. Pentosidine is also an oxidation-dependent AGE structure because its formation is inhibited by anti-oxidative conditions (Baynes 1991). Furthermore, the cellular interactions of proteins modified with AGEs through AGE receptors, such as scavenger receptors and the receptor for AGE (RAGE), are believed to induce several different biological responses, which are involved in the development of diabetic vascular complications. However, since the ligand activity of the AGE-proteins to the scavenger receptors and its pharmacokinetic properties depend on their rate of modification by AGEs, we should carefully prepare the AGE-proteins in vitro and accurately conclude these results to clarify the physiological significance of the interaction between the AGE-receptors and the AGE-proteins.

Glycation of phosphatidylethanolamine (PE) and Amadori-PE formation

In 1993, Bucala et al. (1993) hypothesized that the free amino groups of aminophospholipids are targets for

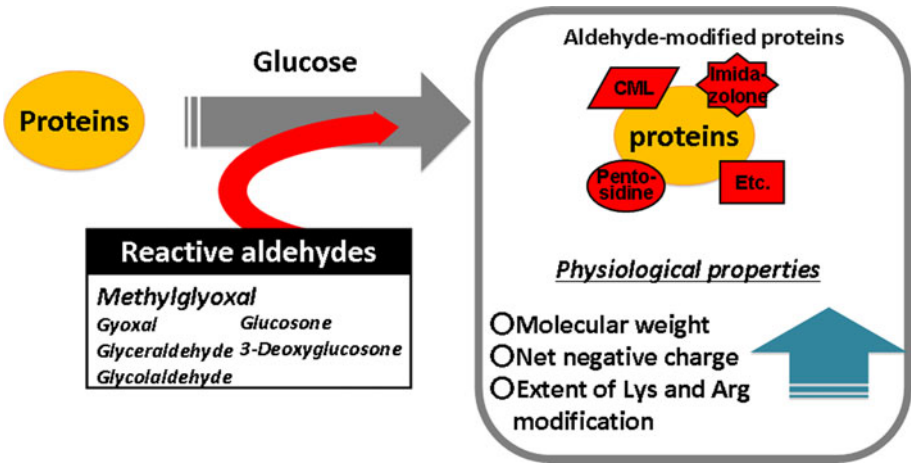

Fig. 2 Change in physicochemical properties of aldehydes-modified proteins

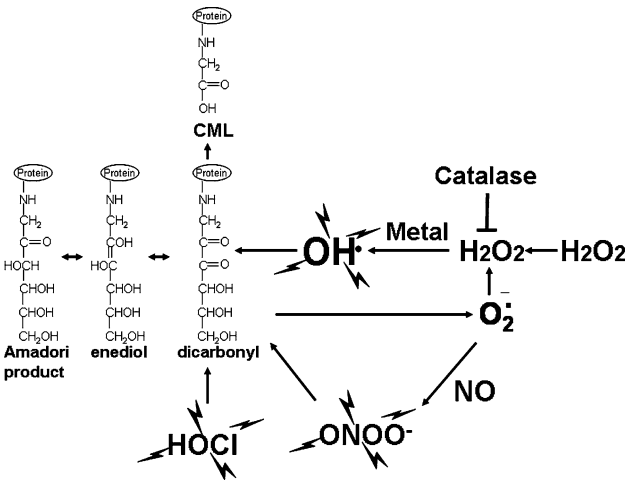

Fig. 3 Proposed mechanism of CML formation

glycation, which expanded the concept of the Maillard reaction into biological systems. Nakagawa et al. (2005) confirmed that PE, but not phosphatidylserine (PS), is exposed to glycation under hyperglycemic conditions, yielding Amadori-PE in vivo. Amadori-PE causes oxidative stress, thus resulting in PCOOH formation in the biomembranes (Oak et al. 2000), thus leading to disordered cellular integrity with angiogenic stimulation (Oak et al. 2003). Amadori-PE and PCOOH may play a key role in the development of diabetes and atherosclerosis. Despite the potential significance of Amadori-PE in pathological signaling, its characteristics and a quantitative method in vivo have not yet been established. Previously, a liquid chromatography method using an ultraviolet (UV)-labeling reagent was developed (Oak et al. 2002). The UV-labeled Amadori products are prepared by the reaction of Amadori-PE with the UV-labeling reagent 3-methyl-2-benzothiazolinone hydrazone (MBTH). The detection limit was 4.5 ng (5 pmol) for PE glycated with glucose and 5.3 ng (5 pmol) for PE glycated with lactose. This allowed us to determine the occurrence of Amadori-PE in several foodstuffs (infant formula and chocolate) and biological samples, such as in 50-week-old aged rat plasma.

There are several reports of Amadori-PE using liquid chromatography with online mass spectrometry (Ravandi et al. 1995) (Utzmann and Lederer 2000). These reports suggested the presence or near-absence of Amadori-PE in blood plasma, red blood cells, and atherosclerotic plaques from human. During the LC-MS analysis, Amadori-PE can only be identified by its molecular ion. In addition, the Amadori-PE peak appears to be concomitant with background contaminants from biological samples. Consequently, to ascertain Amadori-PE generation in vivo, another efficient method is required. A recently developed hybrid quadrupole/linear ion trap (QqLIT) spectrometer, QTRAP, offers special benefit as a liquid chromatography-tandem mass spectrometry (LC-MS/MS) detector for the biomolecular analysis (Houjou et al. 2004). With the advent of QTRAP, both triple quadrupole and ion-trap scans can be performed together in a single step. The product ion scan, neutral loss scan, and multiple reaction monitoring (MRM) can provide useful structural information about the analyte, even in the presence of major background contaminants from complex biological matrices. Based on this knowledge, a QTRAP LC-MS/MS method for Amadori-PE in the plasma of humans with or without diabetes has been developed (Nakagawa et al. 2005). Using this method, it was confirmed that Amadori-PE is present at higher levels in the plasma of diabetic patients compared with healthy subjects, and the possible peroxidative role of Amadori-PE in vivo has been discussed (Nakagawa et al. 2005).

The predominant Amadori-PE species (16:0–18:1 PE, 16:0–20:4 PE, and 16:0–22:6 PE) in the plasma of human subjects with or without diabetes were individually quantified by LC-MS/MS with MRM (Nakagawa et al. 2005). Initially, the parameters were optimized to permit MRM

detection and LC separation of Amadori-PE standards (16:0–18:1 PE, 16:0–20:4 PE, and 16:0–22:6 PE). Thereafter, calibration curves were established by spiking different amounts of Amadori-PE standards into the sample plasma. All calibration curves demonstrated good linearity (0.995–0.999), and the detection limits were 0.1 pmol/injection at a signal-to-noise ratio of 3. In the same way, parameters for LC-MS/MS with MRM could be optimized to detect non-glycated PE standards. Once these conditions were fully optimized, in the MRM chromatograms of plasma of diabetic patients, native PE species (16:0–18:1 PE, 16:0–20:4 PE, and 16:0–22:6 PE) as well as their Amadori products appeared as clear peaks. It was found that the plasma 16:0–18:1 Amadori-PE concentration was below 3 pmol/ml in healthy subjects and was 6–11 pmol/ml in diabetic patients. When calculating the 16:0–18:1 Amadori-PE/16:0–18:1 PE mol%, the amounts of Amadori-PE in the plasma of diabetic patients (0.15 mol%), diabetic patients on chronic hemodialysis (0.29 mol%), and nondiabetic patients on chronic hemodialysis (0.13 mol%) were higher than in the control group (0.08 mol%). Similarly, the average glycation rates of 16:0–20:4 Amadori-PE/16:0–20:4 PE and of 16:0–22:6 Amadori-PE/16:0–22:6 PE in diabetic plasma were 0.13–0.30 mol%, which was higher than in healthy control subjects (0.05–0.10 mol%). The high reproducibility for plasma Amadori-PE/PE mol% (coefficient of variability below 7%) was confirmed and was not altered by the storage of plasma at −80°C for 1 week. It was notable that plasma Amadori-PE levels proportionally correlated with plasma PCOOH as an oxidative stress marker (Nakagawa et al. 2005). Amadori-PE is involved in PE susceptibility to peroxidation. Under hyperglycemic conditions, PE is exposed to glycation, yielding Amadori-PE in the blood, which causes oxidative stress and angiogenic effects. Lipid glycation and lipid peroxidation may be active in the development of diabetes and atherogenesis.

Glycation end products of PE (AGE-PE)

Unlike an early glycation product of PE (Amadori-PE), the occurrence and roles of glycation endproduct of PE (AGE-PE) in vivo have been unclear. Recently, an LC-MS/MS method (Shoji et al. 2010) was developed for the analysis of AGE-PE, such as *N*-carboxymethyl-PE (CM-PE) and *N*-(1-carboxyethyl)-PE (CE-PE). The collision-induced dissociation of CM-PE and CE-PE produced characteristic ions, permitting neutral loss scanning (NLS) and multiple reaction monitoring (MRM) of AGE-PE. According to the findings of an NLS analysis, a series of AGE-PE molecular species was detected in human erythrocytes and blood plasma. In the LC-MS/MS analysis, MRM enables the separation and determination of the predominant AGE-PE species. Between healthy subjects and diabetic patients, no significant differences were observed in the AGE-PE concentrations in erythrocytes and plasma, whereas Amadori-PE concentrations were 3–5 times higher than AGE-PE concentrations in diabetic patients (Table 1). Compared with Amadori-PE, AGE-PE is less likely to be accumulated in the blood of diabetic patients.

Inhibition of PE glycation with pyridoxals

A successful lipid glycation inhibitor has not yet been discovered because of the lack of a lipid glycation model that is useful for inhibitor screening. Recently, a lipid glycation model considering various reaction conditions (glucose concentration, temperature, buffer type, and pH) between PE and glucose was developed (Higuchi et al. 2006). Using the developed model, various protein glycation inhibitors (aminoguanidine, pyridoxamine, and carnosine), antioxidants (ascorbic acid, atocopherol, quercetin, and rutin), and other food compounds (L-lysine, L-cysteine, pyridoxine, pyridoxal, and pyridoxal 5′-phosphate) were evaluated for their antiglycative properties. Pyridoxal 5′-phosphate and pyridoxal (vitamin B6 derivatives) were the most effective antiglycative compounds. These pyridoxals could easily be condensed with PE before the glucose/PE reaction occurred. Because the PE-pyridoxal 5′-phosphate adduct is detectable in human red blood cells and the increased plasma Amadori-PE concentration in streptozotocin (STZ)-induced diabetic rats decreased by the dietary supplementation of pyridoxal 5′-phosphate, it is likely that pyridoxal 5′-phosphate acts as a lipid glycation inhibitor in vivo, which may contribute to diabetes prevention. Interfacing LC with the QTRAP enabled the separation and detection of predominant molecular species of PE pyridoxal 5′-phosphate adduct (reduced forms) in RBCs. The concentration of the PE-pyridoxal 5′-phosphate adduct (16:0–18:2 PE adduct, one of the predominant molecular species) was 12 pmol/ml packed cells. In contrast, the concentration of PE-pyridoxal adduct was below the detection limit (1 pmol/ml packed cells). These findings suggested a new role for pyridoxal 5′-phosphate in preventing lipid glycation in vivo. The dietary pyridoxal 5′-phosphate inhibited lipid glycation in STZ-induced diabetic rats (Higuchi et al. 2006). The amount of Amadori-PE (18:0–22:6-Amdori-PE, a predominant species in plasma) in control rat plasma (STZ-untreated) was an average of 44 pmol/ml. In STZ-induced diabetic rats, a higher concentration of plasma Amadori-PE (18:0–22:6-Amdori-PE) was observed (average 945 pmol/ml). The plasma Amadori-PE concentration is proportional to plasma PCOOH (a marker of oxidative stress; control rats, 8.6 pmol/ml; STZ rats,

Table 1 Lipid glycation products in plasma of healthy subjects and diabetic patients

Molecular species	pmol/ml						nmol/ml	
	CM-PE		CE-PE		Amadori-PE		Native-PE	
	Healthy	Diabetic	Healthy	Diabetic	Healthy	Diabetic	Healthy	Diabetic
16:0–18:1	0.3 ± 0.3	0.2 ± 0.1	<0.1	0.1 ± 0.1	8 ± 6	27 ± 13[a]	0.3 ± 0.2	0.8 ± 0.4[a]
16:0–18:2	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	4 ± 1	9 ± 3[a]	1.3 ± 0.4	2.5 ± 1.0[a]
16:0–20:4	0.6 ± 0.3	0.6 ± 0.3	0.4 ± 0.3	0.6 ± 0.3	39 ± 22	159 ± 75[a]	3.5 ± 1.0	6.0 ± 2.1[a]
16:0–22:6	2.4 ± 2.0	2.5 ± 1.0	0.9 ± 0.5	1.9 ± 0.7	15 ± 7	95 ± 61[a]	25.1 ± 7.1	62.7 ± 17.3[a]
18:0–18:1	0.5 ± 0.2	0.3 ± 0.2	<0.1	<0.1	7 ± 3	25 ± 10[a]	1.0 ± 0.4	1.3 ± 0.5
18:0–18:2	0.9 ± 0.5	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	23 ± 8	72 ± 39[a]	2.9 ± 1.0	4.4 ± 1.9
18:0–20:4	2.9 ± 1.9	1.8 ± 1.1	0.4 ± 0.2	0.5 ± 0.2	64 ± 23	324 ± 183[a]	10.9 ± 3.8	14.3 ± 5.1
18:0–22:6	0.4 ± 0.2	0.9 ± 0.8	<0.1	0.4 ± 0.3	6 ± 2	46 ± 28[a]	10.5 ± 3.7	26.9 ± 8.9[a]
Total	7.7 ± 3.5[b]	6.6 ± 3.1	2.5 ± 1.1	4.2 ± 1.5	165 ± 66	757 ± 377[a]	55.4 ± 15.6	119 ± 33.9[a]
	(0.15 ± 0.07)[c]	(0.06 ± 0.02)	(0.05 ± 0.03)	(0.04 ± 0.01)	(2.97 ± 0.88)	(6.30 ± 1.91[a])		

Values are means ± SD ($n = 8$ for healthy subjects and $n = 10$ for diabetic patients)

[a] $P < 0.01$ compared with healthy subjects

[b] Concentrations of lipid glycation products are presented as sum of molecular species of 16:0–18:1, 16:0–18:2, 16:0–20:4, 16:0–22:6, 18:0–18:1, 18:0–18:2, 18:0–20:4, and 18:0–22:6

[c] Number in parenthesis represents as mmol/mol of total CM-PE, CE-PE, or Amadori-PE species against total native PE species

68.9 pmol/ml). The increments of both plasma Amadori-PE and PCOOH are significantly suppressed, namely by 38 and 39%, respectively, by dietary supplementation of 2 mM pyridoxal 5′-phosphate to STZ rats for 10 weeks. Although no significant differences were observed in plasma lipids between STZ rats and STZ rats fed pyridoxal 5′-phosphate, dietary pyridoxal 5′-phosphate tended to improve the plasma cholesterol and triglyceride concentrations of STZ rats. The pyridoxals have potential as therapeutic compounds for the prevention of diabetes and atherogenesis (Tokita et al. 2005; Asai et al. 2009).

Involvement of glycation with atherosclerosis

The interactions between AGE-modified proteins and the scavenger receptor(s) of macrophages and smooth muscle cells are known to induce the production of several cytokines, such as plasminogen activator and transforming growth factor-beta (Higashi et al. 1997). Furthermore, glycolaldehyde-derived AGE-low-density lipoprotein (AGE-LDL) induces foam cell formation from macrophages (Jinnouchi et al. 1998). These findings suggest that AGE-modified proteins and lipoproteins are involved in the pathogenesis of atherosclerosis.

AGE-proteins are independently prepared in each research group using different protocols, and all groups use excessively high concentrations of glucose and aldehydes. For instance, Higashi et al. prepared highly modified AGE-bovine serum albumin (high-AGE-BSA) by incubating BSA with 1,600 mM glucose for 40 weeks (Higashi et al. 1997), or 33 mM glycolaldehyde for 7 days (Nagai et al. 2000). Schmidt et al. (1992) prepared AGE-BSA by incubating BSA with 250 mM glucose-6-phosphate for 4 weeks, and the cellular interactions of AGEs with RAGE are known to induce several cellular events, including the expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (Schmidt et al. 1995). Vlassara et al. (1992) prepared AGE-BSA by incubating BSA with 50 mM glucose-6-phosphate for 6 weeks and demonstrated that the AGE-receptor 1 (AGER1), a 50-kDa type A integral membrane protein with a short internal domain, suppressed cell oxidant stress and activation signaling via the EGF receptor (Cai et al. 2006). Furthermore, Takeuchi et al. also demonstrated that AGE-BSA prepared using a non-physiologically high concentration of glyceraldehydes resulted in toxicity to the vascular wall cells and the cortical neurons (Takeuchi and Yamagishi 2004). Taken together, these reports demonstrated that the AGE-proteins were prepared with non-physiologically high concentrations of aldehydes and were thereafter employed for cellular experiments. However, Thornalley et al. (2000) demonstrated that end-stage renal disease was associated with a significant increase in the molecular mass of HSA (+255 Da, relative to the control subjects) and that approximately 3% of lysine residues were modified. We prepared mildly modified AGE-BSA (mild-AGE-BSA) by incubating BSA with glucose (50 mM) in a 0.05 M sodium phosphate buffer (pH 7.4) at 37°C for 24 weeks. Our study used a Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOFMS) analysis, and demonstrated that the increased molecular masses of

mild- and high-AGE-BSA were 658 and 9,389 Da, respectively (Nagai et al. 2007). We also compared the content of CML by HPLC between the physiological samples and the AGE-BSA, which was prepared in vitro. For this purpose, human lens proteins were chosen because high amounts of AGE accumulation are observed in long-lived proteins such as the lens proteins. The CML contents for the high- and mild-AGE-BSA were 618.6 mmol CML/mol Lys and 24.4 mmol CML/mol Lys, respectively. However, the CML contents for the diabetic and non-diabetic human lens samples were approximately 17.4 mmol/mol Lys and 8.6 mmol/mol Lys, respectively, therefore indicating that our experimentally-prepared mild-AGE-BSA was already profoundly more modified than the physiological samples. Furthermore, ^{125}I-labeled high-AGE-BSA was specifically associated in a dose-dependent manner to human monocyte-derived macrophages, whereas these changes were not observed using ^{125}I-labeled mild-AGE-BSA. Similar tendencies were observed in CHO cells overexpressing scavenger receptors. Therefore, the ^{125}I-labeled high-AGE-BSA was specifically associated with CHO cells overexpressing SR-BI, CD36, and LOX-1, whereas the association of the ^{125}I-labeled mild-AGE-BSA to these cells was negligible (Nagai et al. 2007) and strongly demonstrated that only the high-AGE-BSA exhibited ligand activity to the scavenger receptors. Taken together, our results demonstrated that the endocytic uptake of physiologically generated AGE-proteins through the scavenger receptors is either negligible or unlikely to occur in vivo, and that AGEs detected inside foam cells in atherosclerotic lesions may have been generated intracellularly rather than representing the endocytic uptake of extracellular AGE-proteins by scavenger receptors.

Nishikawa et al. (2000) demonstrated that the intracellular AGEs content, which was determined by dot-blotting using 6D12, increased when fetal bovine aortic endothelial cells were incubated with 30 mM glucose for 5 days. Therefore, we investigated whether AGEs were generated upon cell injury. For this purpose, human monocytes were incubated with 5 or 30 mM glucose for 1 or 7 days in the presence of 100 nM of phorbol myristate acetate (PMA, for maturation of these cells to macrophages), and we thereafter analyzed the presence of CML-proteins in these cells by a western blot analysis. As shown in Fig. 2, the 65.0 and 65.3 kDa bands, corresponding to CML-modified proteins, were observed by the immunochemical analysis after 7 days of incubation with 30 mM glucose, whereas 25 mM mannitol, used as an osmolality control, in the presence of 5 mM glucose did not result in increased CML. These results suggested that the exposure of macrophages to high glucose levels injurious to the cells resulted in the generation of CML-modified proteins, and that the AGEs detected inside the macrophages in atherosclerotic lesions are generated intracellularly.

As described earlier, CML is generated by the oxidative cleavage of Amadori products by the hydroxyl radical (Nagai et al. 1997), peroxynitrite (Nagai et al. 2002) and hypochlorous acid (Mera et al. 2007). Further studies are therefore required to elucidate the pathways of CML formation inside macrophages. This research may aid in the design of novel strategies for the treatment of diabetic complications and atherosclerosis. Therefore, we should carefully prepare the AGE-proteins in vitro to clarify the physiological significance of the interaction between the AGE-receptors and AGE-proteins.

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