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Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus

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Abstract

Advanced glycation end products (AGEs) are thought to play a major pathogenic role in diabetic retinopathy. The most important AGE is unknown, but as increased serum methylglyoxal-derived hydroimidazolone has been demonstrated in patients with type 2 diabetes mellitus, the aim of the present study was to elucidate possible associations between serum levels of hydroimidazolone and retinopathy in patients with type 2 diabetes mellitus. We recruited 227 patients with type 2 diabetes mellitus and retinopathy ranging from none to proliferative. Level of retinopathy was determined from 7 standard field stereo photographs per eye according to the Early Treatment Diabetic Retinopathy Study. The patients were 66 ± 11 years old, with a known diabetes duration of 14 ± 9 years. Serum levels of hydroimidazolone were determined with a competitive immunoassay. Serum levels of hydroimidazolone were increased in nonproliferative (median, 4.50 U/mL; interquartile range, 3.69-5.77 U/mL) and proliferative retinopathy (median, 4.88 U/mL; interquartile range, 3.70-6.52 U/mL) compared with patients without retinopathy (median, 4.02 U/mL; interquartile range, 3.47-4.88 U/mL) (P = .008 and .002, respectively). There was no association between hydroimidazolone and hemoglobin A_{1c} (r = 0.04, P = .57). In addition, patients with proliferative retinopathy and a relatively short known duration of diabetes, that is, less than the median of 14 years, had increased serum levels of hydroimidazolone (median, 6.91 U/mL; interquartile range, 4.70-8.91 U/mL) compared with those with nonproliferative retinopathy (median, 4.34; interquartile range, 3.86-5.53U/mL, P = .015). Serum levels of hydroimidazolone are increased in type 2 diabetic patients with retinopathy. This association is independent of hitherto known associated factors, such as hemoglobin A_{1c}.

1. Introduction

Diabetic retinopathy is one of the major microvascular complications in both type 1 and type 2 diabetes mellitus as well as the leading cause of blindness among adults of working age [1]. Diabetic retinopathy is closely associated with long-term hyperglycemia [2,3]. How hyperglycemia itself or secondary mechanisms hereof induce retinopathy is not fully understood. Advanced glycation end products (AGEs) can form from nonenzymatic glycation of proteins and are thought to play a major role in the pathogenesis of diabetic retinopathy [4]. However, it is not known which of the many different AGEs is the most important in this respect. In this study, we investigated hydroimidazolone and its association to diabetic retinopathy. Its source, methylglyoxal (MG), is a highly reactive dicarbonyl compound and a product of glycolysis found in all biologic systems. It reacts with arginine, lysine, and cysteine residues of proteins in a reversible manner, whereas it forms AGEs by further irreversible reactions. The AGEs produced accumulate in

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tissue proteins and are increased in diabetes [5]. The main product formed when MG reacts with arginine in proteins is hydroimidazolone (*N*-α-acetyl-*N*-δ-(5-hydro-5-methyl)-4-imidazolone, or MG-H1) that is also quantitatively dominating in vivo. It is also the dominating epitope for detection with our assay and is for simplification referred to as hydroimidazolone.

Increased levels of MG-arginine adducts, mainly hydroimidazolone, have been found in the lens of diabetic patients and in the retina of streptozotocin-induced diabetic experimental rats [6,7]. Increased levels of hydroimidazolone have also been demonstrated in serum of type 2 diabetic patients [8]. To elucidate whether hydroimidazolone levels are increased in human diabetic retinopathy, we studied serum levels of hydroimidazolone in patients with type 2 diabetes mellitus with and without retinopathy.

2. Materials and methods

2.1. Subjects

Two hundred twenty-seven patients with type 2 diabetes mellitus and retinopathy ranging from none to proliferative were selected from the outpatient clinic at the Department of Ophthalmology, University Hospital MAS, Malmö, Sweden. At the time of diagnosis, 221 patients were older than 30 years and 6 patients were younger than 30 years, but were not in need of insulin treatment. The patients gave their informed consent, and the study was approved by the Ethics Committee of Malmö/Lund, Sweden. Patient characteristics are given in Table 1. Blood pressure was measured in the supine position after 5 minutes of rest, using a mercury sphygmomanometer. Diastolic blood pressure was registered at Korotkoff phase V. Venous blood was collected for measurements of hemoglobin A_{1c} (HbA_{1c}) and plasma creatinine. Serum was immediately frozen at −80°C for later analyses of hydroimidazolone. Serum duplicates of all samples were analyzed in random order during 3 consecutive days. Depending on the hydroimidazolone content of the samples, intra-assay coefficient of variation of serum measurements varied from 9% to 15%. The interassay coefficient of variation was 21%. Urinary creatinine and albumin concentrations were measured in a morning urine sample and the albumin-creatinine ratio was calculated.

2.2. Ophthalmologic evaluation

Visual acuity was tested using Early Treatment Diabetic Retinopathy Study (ETDRS) charts. After pupillary dilation, stereo photographs from 7 standard fields were taken of each eye, using a 30° fundus camera (Topcon TRC-50, Tokyo, Japan). Grading was performed in a masked fashion. The patients were characterized according to the ETDRS level of retinopathy in the worse affected eye [9]. Photographs and blood samples were taken at the same visit. If former treatment with panretinal photocoagulation had been given, retinopathy was automatically classified as proliferative, that is, ETDRS level 61.

To study early retinopathy, we separately investigated serum hydroimidazolone levels in patients with diabetes duration below the median of the whole group, which was 14 years, equal to the mean.

2.3. Analytical techniques

HbA_{1c} was analyzed with high-performance liquid chromatography (VARIAN II Hemoglobin A_{1c} program, BioRad, Hercules, CA) (reference range, 4.0%-5.3%) for individuals older than 50 years.

Creatinine in urine was analyzed using a kinetic method (Beckman Synchron LX20, Brea, CA). For analyses of

Table 1
Patient characteristics

	No retinopathy (n = 86)	Retinopathy		*P*
		Nonproliferative retinopathy (n = 89)	Proliferative retinopathy (n = 52)	
Sex (male/female)	41/45	52/37	31/21	
Age (y)	62 (21-86)	68 (39-82)	67 (50-92)	<.001
Age at diabetes diagnosis (y)	53.3 ± 13.1	51.5 ± 10.0	47.4 ± 10.5	.045
Diabetes duration (y)	8.6 ± 6.4	16.8 ± 7.8	19.7 ± 8.2	<.001
Systolic blood pressure (mm Hg)	135 ± 17	140 ± 17	146 ± 18	.001
Diastolic blood pressure (mm Hg)	76 ± 9	78 ± 9	79 ± 8	.033
BMI (kg/m^2)	28.6 ± 4.5	29.0 ± 4.9	28.1 ± 4.9	.94
HbA_{1c} (%)	6.78 ± 1.38	7.51 ± 1.34	7.4 ± 1.32	<.001
Plasma creatinine (μmol/L)	77 (64-88)	88 (73-103)	88 (75-112)	<.001
Urinary albumin-creatinine ratio (mg/mmol)	0.7 (0.5-1.7)	3.9 (1.1-14.1)	4.5 (2.3-23.4)	<.001
Antihyperglycemic treatment (%)				
(a) Diet	12.8	0.0	0.0	<.001
(b) Oral medication	66.3	29.2	15.4	<.001
(c) Insulin	8.1	48.3	59.6	<.001
(d) Combination of b and c (a + b + c + d = 100.0)	12.8	22.5	25.0	.057

Age is given as mean (range). Plasma creatinine and urinary albumin-creatinine ratio are given as median (interquartile range). Other results are given as mean ± SD. P values are from comparisons between no retinopathy and retinopathy.

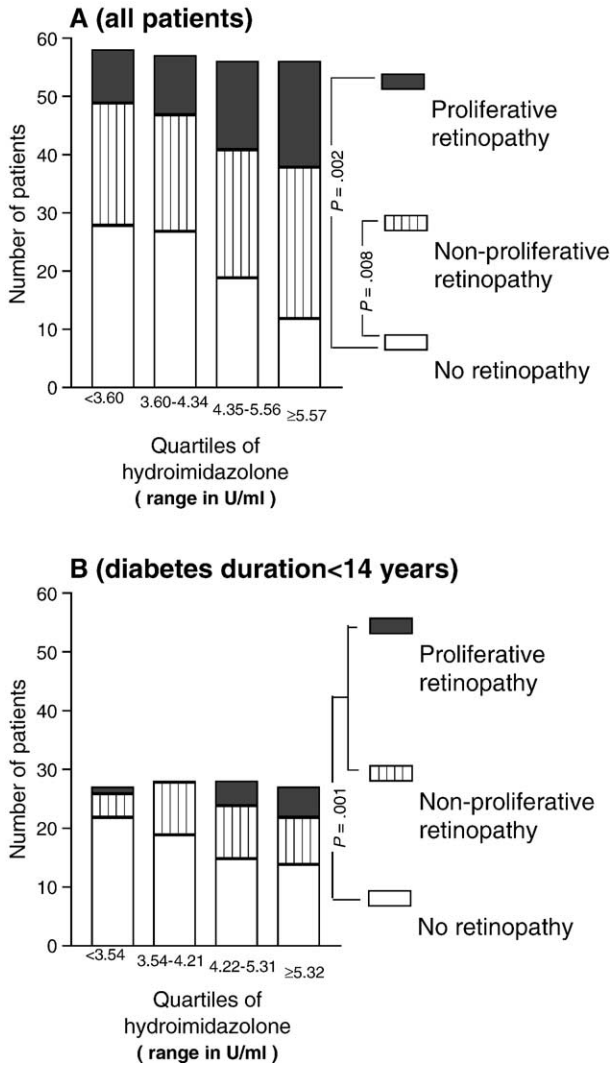

Fig. 1. Distribution of retinopathy related to quartiles of serum hydroimidazolone levels (A) in all patients and (B) in patients with known diabetes duration less than the median of 14 years.

urinary albumin, nephelometry (IMMAGE, Beckman Coulter) or turbidimetry (Beckman Synchron LX20) was used. Normal values for urine albumin–urine creatinine ratio were less than 2.0 mg/mmol for men and less than 2.8 mg/mmol for women. Plasma creatinine was analyzed with a kinetic method (Beckman Synchron LX20). The reference ranges are 51 to 88 and 63 to 105 μmol/L for women and men, respectively.

We have previously developed specific solid-phase, time-resolved competitive immunoassays (Delfia Wallac, Turku, Finland) for determining AGEs in serum [10].

In this study, the serum levels of hydroimidazolone immunoreactivity were determined with the same monoclonal anti–MG-modified arginine antibody and essentially as described by Kilhovd et al [8], except that we coated the microtiter wells in 0.05 mol/L Tris buffer (pH 7.8) and set up our assay in duplicates.

2.4. Statistical methods

All statistical analyses were performed using the SPSS software, version 12.0.0 (SPSS, Chicago, IL). Unadjusted comparisons between patients with and without retinopathy were performed using Student t tests (2-sided) for continuous variables and exact Fisher tests (2-sided) for dichotomous variables. Two-sided Mann-Whitney U test was used when comparing medians of continuous data not normally distributed. Logistic regression analysis, with retinopathy as dependent variable, was used to study the impact of selected variables; hydroimidazolone, plasma creatinine, urinary albumin-creatinine ratio, HbA_{1c}, diabetes duration, age, and blood pressure. These variables were enabled for multiple regression analyses as initial bivariate unadjusted analyses showed significant associations with retinopathy. A significance level of 5% was used.

3. Results

Eighty-nine patients were classified with nonproliferative retinopathy and 52 patients with proliferative retinopathy, whereas 86 had no retinopathy. The serum levels of hydroimidazolone were increased in both nonproliferative (median, 4.50 U/mL; interquartile range, 3.69-5.77 U/mL) and proliferative retinopathy (median, 4.87 U/mL; interquartile range, 3.68-6.64 U/mL) compared with patients without retinopathy (median, 4.02 U/mL; interquartile range, 3.47-4.88 U/mL) (P = .008 and .002, respectively).

By arranging the levels of serum hydroimidazolone in quartiles, a clear relationship between the prevalence of retinopathy and serum hydroimidazolone levels was found (Fig. 1A). In addition, in the subgroup of patients with a known duration of diabetes less than the median of 14 years of the whole group, the serum levels of hydroimidazolone were higher in subjects with retinopathy (median, 4.72 U/mL; interquartile range, 3.97-6.77 U/mL) compared with those without (median, 3.94; interquartile range, 3.43-4.88 U/mL) (P = .001) (Fig. 1B). In this subgroup, too, the highest levels of hydroimidazolone were found in patients with proliferative retinopathy (median, 6.91 U/mL; interquartile range, 4.70-8.91), which was different from levels in patients with nonproliferative retinopathy (median, 4.34 U/mL; interquartile range, 3.86-5.53 U/mL) (P = .015). In the total material, the serum level of hydroimidazolone was weakly correlated with

Table 2
Plasma creatinine intervals with corresponding serum hydroimidazolone levels

Plasma creatinine (μmol/L)	S-Hydroimidazolone (U/mL)	No. of patients
<100	4.3 (2.1-74.0)	172
100-149	4.8 (2.2-13.5)	44
150-200	4.3 (3.2-11.2)	10
>200	7.3 (3.5-9.2)	7

Values are given as median (range). Plasma creatinine was missing for 1 patient.

Table 3
Associations between selected variables and retinopathy

Variables	OR	95% CI	P
Urinary albumin-creatinine ratio	2.68 (+1 quartile)	1.78-4.03	<.01
Hydroimidazolone	1.45 (+1 quartile)	1.01-2.08	.04
HbA_{1c}	1.37 (+1%)	1.00-1.87	<.05
Diabetes duration	1.17 (+1 y)	1.01-1.24	<.01
Age	1.01 (+1 y)	0.97-1.06	.50
Plasma creatinine	1.01 (+1 μmol/L)	0.99-1.03	.17
Systolic blood pressure	1.00 (+1 mm Hg)	0.98-1.03	.96

ORs relate to alterations within parentheses in the "OR" column.

known diabetes duration ($r = 0.13$) and with age ($r = 0.14$) ($P = .04$ for both). Serum levels of hydroimidazolone and HbA_{1c} were not associated ($r = 0.04$, $P = .57$). There was an association between retinopathy and nephropathy as defined by increased plasma creatinine or urinary albumin-creatinine ratio. However, as shown in Table 2, only patients with advanced nephropathy (plasma creatinine levels higher than 200 μmol/L) had increased serum hydroimidazolone levels (median, 7.3 U/mL). Because our primary goal was to investigate possible associations between serum hydroimidazolone and retinopathy, we decided to exclude 7 patients with serum creatinine higher than 200 μmol/L because they all had increased serum hydroimidazolone, most likely due to a decreased kidney function. However, our results were unaffected whether we included them or not. In the remaining patients with plasma creatinine levels less than 200 μmol/L, we found no association between serum hydroimidazolone and serum creatinine.

The association of different variables with retinopathy analyzed by using multiple regression is shown in Table 3, where odds ratios (ORs) relate to alterations within parentheses in the "OR" column. Hydroimidazolone was found to be significantly associated with retinopathy (OR = 1.45, $P = .04$)

4. Discussion

In both nonproliferative and proliferative retinopathy, we observed significantly increased serum levels of the AGE hydroimidazolone compared with patients without retinopathy.

The immunoassay used is quite specific for the major MG adduct formed in vivo (MG-H1) [11]. Approximately 2% of human serum albumin contains MG-H1 residues. Furthermore, among other tissues, retina and plasma proteins in the streptozotocin-induced diabetic rat had increased levels of this hydroimidazolone [11]. Some of the patients in the present study had reduced kidney function. As increased serum hydroimidazolone was revealed only in those with plasma creatinine higher than 200 μmol/L, they were excluded from the study. Furthermore, no association between urinary albumin-creatinine ratio and hydroimidazolone was shown. This means that the association between retinopathy and hydroimidazolone is not confounded by possible diabetic kidney disease in the same patient.

To our knowledge, this is the first clinical study demonstrating an association between hydroimidazolone and human diabetic retinopathy. The pathogenic mechanism in diabetic retinopathy of AGEs in general and hydroimidazolone in particular is not clear. There is a marked AGE accumulation in cultured bovine retinal pericytes [12] and in autopsied human diabetic neural retina [13]. One of the earliest changes observed in retinal microvessels in diabetic retinopathy is the selective loss of intramural pericytes, probably caused by apoptosis [14]. Loss of pericytes may produce decreased capillary tonicity, causing microaneurysms and vessel dilation. Pericytes are also thought to influence regulation of vascular permeability. Recently, a 3-fold increase of pericyte apoptosis has been demonstrated after long-term exposure to MG-modified bovine serum albumin [15].

An alternate hypothesis for pericyte loss is via up-regulation of angiopoietin 2 [16]. The hyperglycemia-induced angiopoietin 2 expression in retinal Müller cells was completely prevented by overexpression of the enzyme glyoxalase I [17], an enzyme known to detoxify MG and glyoxal. This enzymatically controlled removal of MG can result in different MG levels in the presence of similar glucose levels. Individual differences in the efficiency of detoxifying MG may be crucial to the patients' susceptibility for complications. Observations that glyoxalase activity decreases with aging [18] cannot explain our observed association between hydroimidazolone and retinopathy, as this association remained significant after adjusting for age.

Oxidative stress may be induced during the apoptotic process because treatment with various antioxidants completely inhibited pericyte apoptosis in cell culture [15]. Reactive oxygen species might also contribute to the pathogenesis of diabetic retinopathy because administration of antioxidants to alloxan diabetic rats for up to 18 months was found to inhibit the development of characteristic early lesions of retinopathy, such as acellular capillaries and pericyte ghosts [19]. Vascular endothelial growth factor is a central growth factor in both preproliferative and proliferative diabetic retinopathy. Exposing retinal cells in vitro and in vivo to AGEs is known to cause significant up-regulation of vascular endothelial growth factor, although the mechanisms are mainly unknown [20]. AGEs can simultaneously cause significant blood-retinal barrier breakdown in nondiabetic rats infused with AGE-modified proteins [21]. Preformed AGEs can initiate abnormal proliferative responses in both retinal microvascular endothelial cells and human vascular endothelial cells [22]. Aminoguanidine, a well-known scavenger of dicarbonyls such as MG, inhibits the formation of hydroimidazolone [23]. A recent human trial on aminoguanidine showed that fewer patients on this treatment experienced a 3-step or more progression of retinopathy than those treated with placebo [24].

Benfothiamine—a lipid-soluble derivative of thiamine—has been shown to prevent experimental diabetic

retinopathy by blocking 3 major pathways of hyperglycemic damage, including the production of hydroimidazolone intracellularly, measured by the same monoclonal antibody as in the present study [25]. This may indicate that hydroimidazolone is directly involved in the pathogenesis of diabetic retinopathy.

We found a strong and significant association between HbA_{1c} and diabetic retinopathy in the present study ($P < .0001$). However, the association between hydroimidazolone and diabetic retinopathy is independent of HbA_{1c}. In an earlier study [8], we have also shown a lack of association between HbA_{1c} and hydroimidazolone in type 2 diabetes mellitus.

In conclusion, we have shown an association between retinopathy and increased serum levels of hydroimidazolone in type 2 diabetes mellitus. This association was independent from hitherto known associated factors, such as HbA_{1c} and urinary albumin-creatinine ratio. We also observed an association between retinopathy and hydroimidazolone in patients with a comparatively short known duration of diabetes. In this subgroup, *S*-hydroimidazolone levels were also increased as retinopathy became more overt.

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