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Time course of specific AGEs during optimised glycaemic control in type 2 diabetes

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ABSTRACT

Background: Several advanced glycation endproducts (AGEs) are formed in the hyperglycaemic state. Although serum AGEs correlate with average glycaemic control in patients with type 2 diabetes and predict the development of complications, it is not known how serum AGEs change during optimisation of diabetes therapy.

Methods: We evaluated the change in serum levels of total AGE and the AGEs CML (N^{ε}-carboxymethyllysine) and MGHI (methylglyoxal-derived hydroimidazolone), as well as markers of endothelial function in 28 subjects with type 2 diabetes, who were poorly controlled on oral agents, before and after the institution of insulin therapy.

Results: Mean subject age (± SEM) was 58 ± 2 years, body mass index 27.7 ± 0.8 kg/m², and known duration of diabetes was 8.1 ± 0.9 years. With insulin treatment fasting blood glucose levels dropped from 12.1 ± 0.9 mmol/l to 6.9 ± 0.3 and 8.1 ± 0.4 mmol/l after three and six months, respectively (both p<0.001), while HbA_{1c} decreased from 10.0 ± 0.3 to 7.8 ± 0.2% (p<0.001). Endothelial function improved as indicated by a small but significant decrease in soluble intercellular cell adhesion molecule (sICAM-1) (152 ± 10 to 143 ± 8 ng/ml, p<0.02) and sE-selectin (111 ± 16 to 102 ± 12 ng/ml, p<0.02) levels. In contrast, we observed only a tendency towards a decrease in CML levels (110 ± 22 to 86 ± 13 µg/mg protein, p=ns), but a small increase of MGHI (from 0.23 ± 0.02 to 0.29 ± 0.04 U/mg protein, p<0.02). At baseline, 16 patients were on metformin, which is known to reduce methylglyoxal levels and reduce generation of reactive oxygen species. They had similar levels of CML and MGHI to the 12 non-metformin users, although their HbA_{1c} was lower (9.4 ± 0.3 *vs* 10.7 ± 0.6 %). During insulin, patients receiving concomitant metformin therapy showed a similar course of CML and MGHI to those not taking metformin.

Conclusion: Although insulin therapy improved HbA_{1c} and markers of endothelial function, the levels of serum AGEs did not follow the same time course. This suggests that these specific AGEs are influenced by other factors in addition to overall glycaemia, such as oxidative stress.

KEYWORDS

Adhesion molecules, AGE, endothelial dysfunction, insulin therapy, methylglyoxal-derived hydroimidazolone, N^{ε}-carboxymethyllysine, type 2 diabetes

INTRODUCTION

Hyperglycaemia is a major factor responsible for the development of diabetic complications. In recent years, several studies have reported on the effects of intensive glucose-lowering therapy in preventing both microvascular and macrovascular complications. Two of these major trials were the United Kingdom Prospective Diabetes Study (UKPDS) and the Diabetes Control and Complications Trial (DCCT).[1-3] It was clearly shown that optimised diabetes treatment resulted in a reduction in the development and progression of microvascular complications in both type 2 and type 1 diabetic patients, and to a lesser extent of macrovascular disease.

The biochemical changes induced by hyperglycaemia are complex and several mechanisms are involved,[4] including the polyol pathway, activation of protein kinase C,

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increased oxidative stress and the formation of advanced glycation endproducts (AGEs).

AGE formation results from the reaction of a carbonyl group of a reducing sugar with the free amino groups of a protein. Known as Schiff's base, this will react further to an Amadori product which rearranges to the AGEs.[5,6] This glycation process leads to the formation of a group of heterogeneous components which are associated with several pathological processes in patients with diabetes.[7] Next to this glycation process, lipid peroxidation or oxidative stress in general can lead to the formation of reactive carbonyl compounds (methylglyoxal, glyoxal or 3-deoxyglucosone), which can react with proteins thus forming advanced lipid peroxidation endproducts or advanced lipoxidation endproducts (ALEs), respectively.[8] Circulating AGEs can react with their receptors and induce several cellular responses in the vessel wall, such as formation and activation of cytokines and increased expression of adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by the endothelium.[9-11] Endothelial dysfunction plays an important role in the development of long-term diabetic complications,[12] and it has been shown *in vivo* as well as *in vitro* that these adhesion molecules correlate well with endothelial dysfunction.[13-18]

It is expected that intensive glucose-lowering therapy will lead to a gradual reduction in the formation of AGEs, as was shown for haemoglobin AGE several years ago.[19] Although serum AGEs correlate with average glycaemic control and predict the development of complications, it is not known how other specific serum AGEs change during optimisation of diabetes therapy.[20]

In this study we assessed the metabolic effects of improved glucose control by institution of insulin therapy in subjects with type 2 diabetes, who failed to respond adequately to oral blood glucose-lowering therapy. We followed the changes of specific AGEs over time, their interaction and studied whether improvement in glycaemic control resulted in an improved endothelial function indicated by the levels of circulating adhesion molecules.

METHODS AND MATERIALS

Subjects and study design

Twenty-eight subjects (14 males, 14 females) with type 2 diabetes, who were insufficiently controlled on oral glucose-lowering medication (sulphonylurea with or without metformin) and therefore started insulin therapy, participated in this study. Their mean age (± SEM) was 58 ± 2 years, body mass index 27.7 ± 0.8 kg/m^2, and known duration of diabetes was 8.1 ± 0.9 years. Subjects were attending the outpatient departments of four Dutch hospitals, Maastricht University Hospital, Radboud University Nijmegen Medical Centre, and the hospitals of Boxmeer and Leidschendam. They were all participating in a larger international study assessing the effects of therapy with a new recombinant human insulin preparation (Sanofi-Aventis). Men and women between the age of 30 and 80 years were included in this study if they had documented type 2 diabetes, had normal kidney function, and failed to achieve adequate glycaemic control on oral blood glucose lowering agents. Written informed consent was obtained from all participants.

At the start of the study subjects were screened and demographic data as well as fasting blood samples were taken. Data on concomitant medication were obtained throughout the study. Nine patients were taking an ACE inhibitor, six patients a β-blocker, while eight patients were treated with a statin and two patients were on acetylsalicylic acid. These treatments were not altered during the study.

The choice for a specific insulin regimen was made by the treating physician for the individual patient, and based on the results of home blood glucose monitoring. Patients were seen at regular intervals in the outpatient clinic to monitor insulin therapy, and the insulin dose was adjusted by the treating physician on the basis of home blood glucose monitoring. For the purpose of the study, fasting blood samples were taken at baseline, i.e. before insulin therapy, and three and six months after initiation of insulin therapy. On these occasions, blood samples were directly centrifuged and serum was stored in small aliquots at -80°C until further analysis.

Methods

HbA_{1c} levels were measured with HPLC (Bio-Rad Variant II,Hercules CA). The nondiabetic reference range was 4.2 to 6.5%, and the assay was linear up to 17.9%. Fasting blood glucose was measured by a hexokinase method (Olympus, Southall, UK).

Measurements of the adhesion molecules (sE-selectin and sICAM-1) were performed with an ELISA.[21] sICAM-1 standard was obtained from Bender MedSystems (Vienna, Austria); sE-selectin standard was prepared as described elsewhere.[21]

Serum total AGE levels were determined with a polyclonal antibody raised against AGE RNase by the DELFIA method.[22] One AGE unit was defined according to Makita *et al.* as the competitive activity of 1 μg AGE-BSA standard.[23] The final serum concentration of AGEs was corrected for total protein concentration in each serum sample in the following equation [AGE, U/ml] x [sample protein/mean protein concentration of all sera measured]. All analyses were performed in the same run. Methylglyoxal-derived hydroimidazolone (MGHI) levels were determined using a method similar to the total AGE

measurement.[24] One hydroimidazolone unit was defined as the competitive activity of 1 μg of MG-modified BSA standard. The serum concentration of hydroimidazolone was adjusted for the total protein concentration in each sample, and is expressed as U/mg protein. In this way, possible systematic errors due to the differences in protein content between groups were avoided.

N^{ε}-carboxymethyllysine (CML) was measured using a newly developed HPLC method.[25] CML data were then normalised against plasma protein concentration resulting in the final concentration of ng CML/mg plasma protein.

Statistics

At baseline several patients were on metformin. Since this was an open study, some subjects continued this medication concomitantly with their insulin treatment, while others stopped all oral agents and switched to insulin alone. As it is known that metformin may scavenge intermediate glycation products such as methylglyoxal,[26] the results of insulin therapy were analysed in the whole group, and post-hoc for metformin users and non-users separately. Also the observed changes were compared between patients who showed a good improvement in metabolic control (decrease in HbA_{1c} of >1.5%: good responders), and those with only moderate changes (decrease in HbA_{1c} of ≤1.5%; poor responders).

All results were expressed as means ± SEM. Data were analysed using one-way ANOVA, paired t-tests and Pearson correlations. Statistical analysis was performed using SPSS 10.0, SPSS, Chicago, Il, USA. P values <0.05 were considered to indicate statistical significance.

RESULTS

At baseline metabolic control was insufficient, indicated by mean HbA_{1c} levels of 10.0 ± 0.3 % and fasting blood glucose (FBG) levels of 12.1 ± 0.9 mmol/l. Insulin therapy resulted in a significant improvement in glycaemic control: HbA_{1c} in the total group decreased to 7.8 ± 0.2 % at three months with no additional change after six months (*table 1*). Mean daily insulin dose was 39 ± 5 U at six months, and at that time four subjects were using one daily insulin injection, 16 used a mixture of fast-acting and neutral protamine Hagedorn (NPH) insulin twice daily, and eight were on a four-injection regimen comprising fast-acting insulin before meals and NPH insulin at bedtime. The serum levels of the adhesion molecules E-selectin and ICAM-1 also decreased significantly, indicating an improvement in endothelial function. It appeared that total AGE serum levels did not change significantly after six months. We observed a small decrease in CML levels, which was not statistically significant, while levels of MGHI increased significantly (*table 1*). The course of HbA_{1c} and CML in the individual patients is depicted in *figure 1*.

Overall, HbA_{1c} levels were not correlated with serum AGE levels (total AGE, CML and MGHI) nor with serum adhesion molecule levels (E-selectin and ICAM-1). Total AGE and CML did correlate with sE-selectin ($R_{Pearson}$=0.36, p=0.013 and $R_{Pearson}$=0.29, respectively, p=0.004), but not with sICAM-1.

Influence of metformin use

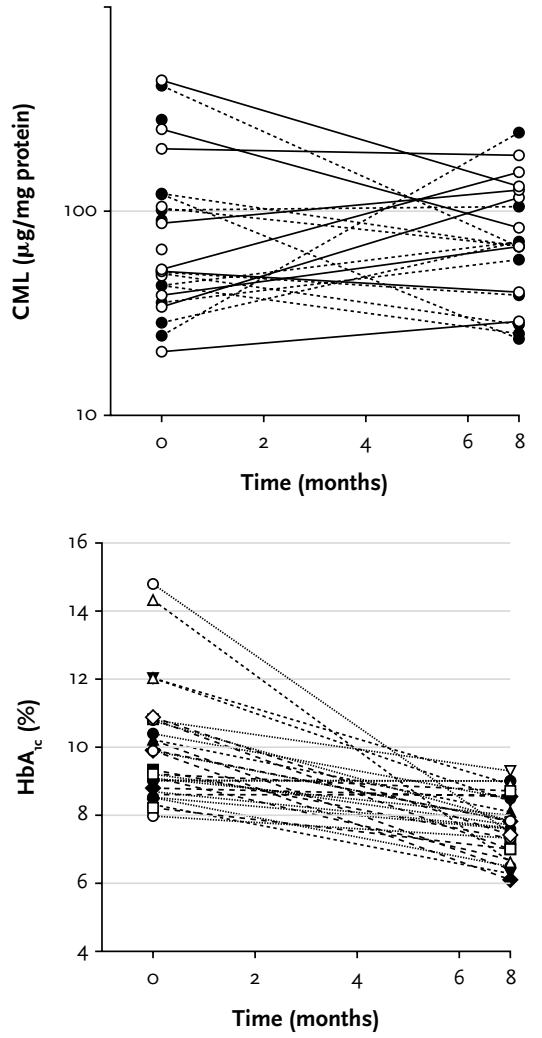
The relevant laboratory parameters at baseline were compared between subjects who were only on sulphonylurea (SU) and those taking SU with metformin. As shown in *table 2*, FBG and HbA_{1c} were significantly lower in metformin users, although their diabetes duration was longer. No differences were observed in the levels of adhesion molecules or the different AGE measurements. Of the 16 metformin users, six continued this drug during insulin therapy, and ten switched to therapy with insulin alone. The decrease in HbA_{1c} in subjects who continued metformin in addition to insulin

Table 1. *Changes in the main variables during the course of the study, after initiation of insulin therapy at baseline*

	Baseline	Three months	Six months
FBG (mmol/l)	12.1 ± 0.9	6.9 ± 0.3[#]	8.1 ± 0.4[#]
HbA1c (%)	10.0 ± 0.3	7.8 ± 0.2[#]	7.8 ± 0.2[#]
Insulin dose (U/day)	-	38 ± 4	39 ± 5
sICAM-1 (ng/ml)	152 ± 10	143 ± 8[†]	147 ± 8[†]
sE-selectin (ng/ml)	111 ± 16	96 ± 12[†]	102 ± 14[†]
Total AGE (U/ml)	6.7 ± 0.7	6.7 ± 0.5	7.4 ± 1.0
MGHI (U/mg protein)	0.23 ± 0.03	0.23 ± 0.02	0.29 ± 0.04[†]
CML (ng/mg protein)	114 ± 23	126 ± 18	86 ± 13[$]

[#]p<0.001 *vs* baseline; [†]p<0.02 *vs* baseline; [$]p=0.086.
FBG = fasting blood glucose; MGHI = methylglyoxal-modified hydroimidazolone; CML = N^{ε}-carboxymethyllysine.

Figure 1. *Course of HbA_{1c} and CML in the individual patients during the study period*



Different symbols depict different patients.

treatment was 1.7% (from 9.3 ± 0.4 to 7.6 ± 0.4%), which was comparable with subjects who switched to insulin alone 1.3% (from 9.4 ± 0.4 to 8.1 ± 0.4%). There were, however, considerable differences in insulin dose: the metformin users injected 18 ± 3 U of insulin per day at six months, while subjects on insulin alone used 45 ± 5 U daily (p=0.009). We were unable to demonstrate significant differences in the changes of the various AGE levels between metformin users and nonusers, but the group sizes may be too small..

Influence of insulin efficiency

As insulin therapy has a variable effect on glycaemic control in patients, we assessed whether the observed changes were related to the efficiency of insulin therapy. Poor responders were at better metabolic control at baseline (HbA_{1c} 8.9 ± 0.2 *vs* 10.7 ± 0.5%), but had a longer duration of diabetes (*table 3*). Baseline levels of total AGE, MGHI and CML were not different between the groups. At six months no significant difference could be seen between the two groups, their insulin dose was identical, and the change in total AGE, MGHI, CML and adhesion molecule levels over time was not significantly different between the two groups.

DISCUSSION

It has been suggested that the formation of AGEs plays an important role in the development of microvascular and macrovascular complications in patients with diabetes mellitus, and the level of glycated haemoglobin (HbA_{1c}) is a major predictor. However, it is not known how serum AGEs change during optimisation of diabetes therapy. In the present study we observed that insulin therapy improved glycaemic control after three and six months

Table 2. *Relevant laboratory parameters at baseline according to use of metformin*

	Metformin + SU	SU only
Gender (male/female)	7/9	7/5
Age (years)	60 ± 2	55 ± 3
BMI (kg/m²)	27.0 ± 0.8	29.0 ± 1.5
Known diabetes duration (years)	10.1 ± 1.1	5.3 ± 1.0#
Fasting blood glucose (mmol/l)	10.5 ± 0.7	14.2 ± 1.6#
HbA1c (%)	9.4 ± 0.3	10.7 ± 0.6#
sICAM-1 (ng/ml)	153 ± 14	150 ± 15
sE-selectin (ng/ml)	98 ± 19	127 ± 29
Total AGE (U/ml)	6.2 ± 0.9	7.3 ± 0.9
MGHI (U/mg protein)	0.25 ± 0.05	0.21 ± 0.03
CML (ng/mg protein)	109 ± 31	112 ± 32

#p<0.05 *vs* metformin + SU group.
SU = sulphonylurea; BMI = body mass index; MGHI = methylglyoxal-modified hydroimidazolone; CML = N^{ε}-carboxymethyllysine.

as indicated by a considerable decrease in both FBG and HbA_{1c}.[3] However, this improvement of glycaemic control did not result in a significant decrease in total AGE levels. A slight but not significant decrease in CML levels was observed, while MGHI even increased significantly.

In addition to the insulin therapy, several patients received concomitant medication such as metformin. The UKPDS has shown that metformin treatment in obese type 2 diabetic patients reduced cardiovascular complications to a greater extent than could be expected from its glucose-lowering potential.[27,28] This resulted in the hypothesis that metformin also interacts with another molecular mechanism resulting from hyperglycaemia. Several studies [26,29] have shown that metformin plays an important role in inhibiting dicarbonyl-mediated AGE formation and accumulation, which should be reflected in decreased MGHI[24] and CML levels. At baseline, metformin users had a lower HbA_{1c} and fasting blood glucose levels than nonusers. However, in this small group of patients we observed no differences in total AGE, MGHI or CML levels. Of the 16 metformin users, six subjects continued this medication in addition to insulin. No different changes in the levels of HbA_{1c}, fasting blood glucose or the various AGE measurements were seen.

Thus it is apparent that HbA_{1c} and the levels of AGEs did not follow the same time course. This suggests that these specific AGEs, such as CML and MGHI, are influenced by other factors in addition to overall glycaemia, and that these factors are of greater importance in determining AGE levels. It has been suggested that CML and MGHI in serum may also be derived from lipid peroxidation[8] or be formed as a consequence of the generation of reactive oxygen species.[9] Again this may relate to changes in metformin treatment. It has been shown that metformin may also decrease production of reactive oxygen species.[30,31] In addition, elevated methylglyoxal levels, increasing after withdrawal of metformin, may directly increase oxidative stress, as was demonstrated *in vitro* in vascular smooth muscle cells.[32]

Furthermore, increased oxidative stress can be generated by postprandial blood glucose excursions, occurring in diabetic patients even when on good metabolic control.[33]

Several studies have shown the potential use of AGEs as marker for the progression and severity of diabetic complication independent from markers for hyperglycaemia such as HbA_{1c}. The discrepancy in the changes in HbA_{1c} and AGEs after insulin treatment supports the notion that they are independent determinants of prognosis.[34,35]

In addition to the time course of AGEs, we measured serum levels of ICAM-1 and E-selectin as an estimate of endothelial function.[36] Insulin therapy resulted in a slight, but consistent and statistically significant decrease of both adhesion molecules, which indicates an improvement in endothelial function. Baseline levels of total AGEs correlated with E-selectin, but not with ICAM-1 levels. It has previously been suggested that AGEs induce the upregulation of adhesion molecules.[13,15] Since we observed that the decrease in adhesion molecules was not paralleled by a decrease in AGE levels, this suggests that other (glycaemic or nonglycaemic) factors add to the effect of AGEs in upregulation of adhesion molecule expression. As receptor for AGE (RAGE) is a multiligand member of the immunoglobulin super family of cell surface molecules, other ligands can interact with RAGE resulting in the same effect as activation of intracellular signalling pathways such as MAP-kinases and NF-κB and the resulting upregulation of adhesion molecules.

Table 3. *Characteristics of subjects responding or not responding to insulin therapy*

	Poor responders (n=12)		Good responders (n=16)	
	Baseline	**Six months**	**Baseline**	**Six months**
Gender (male/female)	5/7	-	9/7	-
Diabetes duration (years)	11.1 ± 1.2	-	5.8 ± 0.9#	-
FBG (mmol/l)	9.5 ± 0.7	8.1 ± 0.7	14.3 ± 1.2#	8.1 ± 0.5
HbA_{1c} (%)	8.9 ± 0.2	8.3 ± 0.3	10.7 ± 0.5#	7.3 ± 0.2#
Insulin dose (U/day)	-	40 ± 7	-	39 ± 6
sICAM-1 (ng/ml)	146 ± 18	158 ± 16	155 ± 12	142 ± 10†
sE-selectin (ng/ml)	98 ± 23	109 ± 29	120 ± 23	99 ± 16†
CML (ng/mg protein)	90 ± 21	73 ± 22	124 ± 34	92 ± 16
MGHI (U/mg protein)	0.26 ± 0.05	0.33 ± 0.08	0.20 ± 0.03	0.26 ± 0.04
Total AGE (U/ml)	6.1 ± 0.9	9.5 ± 1.8	7.2 ± 0.9	6.5 ± 0.6

#p<0.05 *vs* poor responders; †p<0.05 *vs* baseline.
FBG = fasting blood glucose; MGHI = methylglyoxal-modified hydroimidazolone; CML = N^{ε}-carboxymethyllysine.
Poor responders were considered those subjects in whom HbA_{1c} decreased by ≤1.5% (at six months compared with start of the study) and good responders were those individuals in whom a decrease in HbA_{1c} of >1.5% was observed.

To assess the efficiency of insulin therapy on both endothelial function and AGE levels, patients were divided in two groups. Poor responders were considered those subjects in whose HbA_{1c} decreased by ≤1.5% (at seven months compared with start of the study) and good responders were those individuals in whom a decrease in HbA_{1c} of >1.5% was found. Good responders did achieve an average HbA_{1c} of 7.3%, whereas in poor responders HbA_{1c} after six months was 8.3%. Endothelial function, total AGE, CML and MG-derived hydroimidazolone showed no significant differences at the different time intervals, nor a significant different increase or decrease over time. Insulin therapy has a varying efficiency in different patients resulting in different levels of glycaemic control. However, this variance in glycaemic control did not result in a difference in the underlying molecular mechanisms as AGE production and accumulation and the resulting change in adhesion molecule levels.

We conclude that improvement in glycaemic control by glucose-lowering therapy ameliorates endothelial function as assessed by sICAM-1 and sE-selectin levels. Although insulin therapy improved HbA_{1c}, the levels of serum AGEs, CML and MGHI did not follow the same time course. This indicates that these AGEs are formed in different pathways and improving glycaemic control in diabetic subjects will not automatically lead to a reduction in AGE levels. This suggests that their presence in serum is influenced by other factors in addition to overall glycaemia, such as lipid peroxidation or in general oxidative stress. The influence of specific treatment, as metformin, may be of significance as well, since this drug can scavenge methylglyoxal and reduce generation of reactive oxygen species.

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