CLINICAL STUDY

Improved glycemic control induced by both metformin and repaglinide is associated with a reduction in blood levels of 3-deoxyglucosone in nonobese patients with type 2 diabetes

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Abstract

Objective: Metformin has been reported to reduce α -dicarbonyls, which are known to contribute to diabetic complications. It is unclear whether this is due to direct quenching of α -dicarbonyls or to an improvement in glycemic control. We therefore compared the effects of metformin versus repaglinide, an antihyperglycemic agent with an insulin-secreting mechanism, on the levels of the α -dicarbonyl 3-deoxyglucosone (3DG).

Methods: We conducted a single-center, double-masked, double-dummy, crossover study involving 96 nonobese patients with type 2 diabetes. After a 1-month run-in on diet-only treatment, patients were randomized to either repaglinide (6 mg daily) followed by metformin (2 g daily) or vice versa each during 4 months with a 1-month washout between interventions.

Results: 3DG levels decreased after both metformin (-19.3% (95% confidence interval (CI): -23.5 , -14.8)) and repaglinide (-20.8% (95% CI: -24.9 , -16.3)) treatments, but no difference was found between treatments (1.8% (95% CI: -3.8 , 7.8)). Regardless of the treatment, changes in glycemic variables were associated with changes in 3DG. Specifically, 3DG decreased by 22.7% (95% CI: 19.0 , 26.5) per s.d. decrease in fasting plasma glucose (PG), by 20.0% (95% CI: 16.2 , 23.9) per s.d. decrease in seven-point mean plasma glucose, by 22.5% (95% CI: 18.6 , 26.6) per s.d. decrease in area under the curve for PG, by 17.2% (95% CI: 13.8 , 20.6) per s.d. decrease in HbA_{1c}, and by 10.9% (95% CI: 6.4 , 15.5) per s.d. decrease in Amadori albumin. In addition, decreases in 3DG were associated with decreases in advanced glycation endproducts and endothelial markers.

Conclusion: Improved glycemic control induced by both metformin and repaglinide is associated with a reduction in 3DG levels in nonobese individuals with type 2 diabetes. This may constitute a shared metabolic pathway through which both treatments have a beneficial impact on the cardiovascular risk.

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Introduction

The mechanisms underlying the development of vascular complications in diabetes are not completely understood. A potential mechanism by which hyperglycemia and its immediate biochemical sequelae induce vascular complications involves the increased formation of advanced glycation endproducts (AGEs) (1).

The deleterious effects of AGEs are attributed to direct modification of extracellular matrix proteins, resulting in collagen cross-links, which lead to increased vascular stiffness (1). In addition, binding of AGEs to a variety of cell-surface receptors, such as the receptor for AGEs, triggers signal transduction pathways and the production of reactive oxygen species, resulting in proinflammatory cellular responses (2). Given these

deleterious effects of AGEs, several potential inhibitors (e.g. aminoguanidine, pyridoxamine, and cross-link breaker ALT-711) have been developed to reduce the clinical impact of AGEs (3).

Although the formation of AGEs is complex, it is well established that α -dicarbonyl compounds such as 3-deoxyglucosone (3DG) and methylglyoxal are reactive intermediates in the formation of AGEs (4). The levels of 3DG have been shown to be elevated in patients with diabetes (5, 6), particularly in those with diabetic microangiopathy, such as retinopathy (5), neuropathy (5), and nephropathy (5, 7).

Aminoguanidine, which is a small hydrazine-like molecule, inhibits the formation of AGEs by interaction with and quenching of dicarbonyl compounds (8). Metformin, an antihyperglycemic agent, which enhances insulin sensitivity and lowers hepatic glucose output, may also have antiglycation properties, with a working mechanism similar to aminoguanidine due to a similarity in structure between both the agents (9–11). The observation of reduced levels of methylglyoxal in patients using metformin as compared with controls supports this view (9). Based on this additional effect, the beneficial impact of metformin on cardiovascular complications of type 2 diabetes, as for instance reported in the UKPDS study (12), could be, at least in part, ascribed to its specific effects on detoxifying dicarbonyl compounds, thereby preventing AGE accumulation. However, a reduction in dicarbonyls following treatment with metformin may also derive from an improvement in glycemic control.

In view of these considerations, and in order to disentangle those two possible operating mechanisms, we have investigated, in a single-center, double-masked, double-dummy, crossover study in nonobese patients with type 2 diabetes (initially investigating the hypothesis of equivalent antihyperglycemic effects of metformin and repaglinide; ClinicalTrials.gov Identifier: NCT00118950) (13–15)) i) the effects of metformin versus repaglinide – an antihyperglycemic agent with a different working mechanism (i.e. stimulating insulin secretion) – on the levels of 3DG; ii) the associations of treatment-induced changes in glycemic variables (i.e. fasting plasma glucose (PG), seven-point mean PG, area under the curve for PG (AUC-PG), HbA_{1c}, and Amadori albumin) with changes in 3DG levels; and iii) the associations between changes in 3DG levels and changes in biomarkers of advanced glycation, endothelial dysfunction, and low-grade inflammation.

Materials and methods

Study design

In 2001–2003, we conducted a single-center, randomized, double-masked, double-dummy, crossover study with 96 nonobese (body mass index ≤ 27 kg/m²)

insulin-naïve individuals with type 2 diabetes as described in detail elsewhere (13–15). Briefly, we included 96 subjects who had type 2 diabetes diagnosed when 40 years of age or older, had no history of ketonuria, or diabetic ketoacidosis and, at enrollment, presented with a fasting serum C-peptide ≥ 300 pmol/l or a nonfasting or glucagon-stimulated C-peptide ≥ 600 pmol/l. After enrollment, patients stopped intake of oral hypoglycemic agents and underwent a 1-month run-in period on diet-only treatment. After the run-in period, patients were randomized to receive either 2 mg repaglinide three times daily plus placebo metformin followed by 1 g metformin twice daily plus placebo repaglinide, or vice versa, each treatment for a period of 4 months with a 1-month washout period between interventions.

Out of the 96 included patients, 93 and 89 patients initiated a treatment period with metformin and repaglinide respectively. Throughout the course of treatments, 20 patients (21%) dropped out, leaving 76 patients (79%) who completed the whole trial; 83 and 82 patients completed a treatment period with metformin or repaglinide respectively. Ten (11%) patients dropped out during metformin treatment, seven (8%) during repaglinide treatment, and three (3%) during the washout period; one after repaglinide and two after metformin treatment (Fig. 1) (14).

Results regarding the effects of treatments on trial's primary endpoint, HbA_{1c}, and secondary endpoints, such as postprandial glucose and lipid metabolism, as well as markers of inflammation and endothelial dysfunction have been reported previously (13–15). This study focuses specifically on the effects of treatments on blood levels of 3DG, which due to the complexity of its assessment only became available recently. Measures to ensure proper blood sampling to enable future assessment of 3DG levels were planned prior to and undertaken during the trial (see also later).

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Copenhagen County, Denmark.

Blood sampling

Patients were instructed to obtain seven-point home-monitored PG measurements (preprandial, 90 min postprandial, and prebedtime) 2 days per week during the treatment periods, which were used to calculate the mean PG levels (seven-point MPG).

Patients were further investigated at the Steno Diabetes Center (Gentofte, Denmark) where blood samples were taken during the last week before the patient entered a treatment period and on the last day of each treatment period. Blood samples were drawn with minimal venous occlusion between 0800 and 0900 h after a 10 h overnight fast and 25 min of supine rest. Additionally, during a standardized 0–6 h meal test, blood was withdrawn at $t=0$, 90, 180, 270, and 360 min, and used to calculate the AUC-PG

Figure 1 Study design and flowchart of the 96 randomized patients.

by the trapezoidal rule (i.e. $1.5 \text{ h} \times (0.5 \times \text{concentration}_{t=0 \text{ h}} + \text{concentration}_{t=1.5 \text{ h}} + \text{concentration}_{t=3.0 \text{ h}} + \text{concentration}_{t=4.5 \text{ h}} + 0.5 \times \text{concentration}_{t=6.0 \text{ h}})$ in $\text{mmol/l} \times \text{h}$) (13).

Sodium citrate and EDTA plasma and serum samples were prepared by centrifugation at 2000 *g* for 10 min at 20 °C and immediately frozen hereafter. Samples were stored at −80 °C until analyses.

α -dicarbonyls (such as 3DG) react rapidly with arginine and lysine residues in proteins. Therefore, as the measurement of 3DG was anticipated prior to initiating the trial, 0.5 ml whole blood was collected in specific blood collection tubes to be immediately mixed with 1.0 ml of 1.2 mol/l perchloric acid to precipitate the protein fraction and stored at −80 °C until assayed.

Biochemical analyses

3DG was measured using liquid chromatography–tandem mass spectrometry (LC–tandem MS) (6). After thawing the acidified blood samples, the samples were centrifuged (5 min at 20 000 *g*) and 80 μl of the

supernatant was mixed with 100 μl of the internal standard solution (1 $\mu\text{M/l}$ 2,3-pentanedione dissolved in ethanol). After the addition of 20 μl of the derivatization reagent (10 mM/l 2,4-dinitrophenylhydrazine dissolved in 1.2 M/l perchloric acid), the samples were incubated for 16 h at room temperature. Chromatographic separation was performed on an Xterra MS C18 column (4.6 $\times$ 50 mm, 3.5 μm particle size) (Waters, Milford, MA, USA) using a linear gradient from 40 to 95% acetonitrile in water over 9 min at a flow rate of 1 ml/min. Mass transitions of 521.1–431.0 and 521.1–182.1 for 3DG and 459.1–182.1 for the internal standard were monitored in negative ion mode. The derivatization and the chromatographic separation were performed at a low pH, minimizing the chance of 3DG formation from sugars and fructosyl-lysine at this stage (4). To test the recovery of 3DG, calibration curves prepared in whole blood (two different samples) were compared with a calibration curve prepared in water. The slopes of the calibration curves in whole blood were not significantly different from the slope of the calibration curve in water. As part of validation of the method, we performed a comparison with a previously published method, based on derivatization with 1,2-diamino-4,5-dimethoxybenzene (DDB) followed by HPLC analysis with fluorescence detection (16). In a comparison of perchloric acid supernatants of 44 whole blood samples, results obtained with both methods were highly correlated ($r=0.92$; $P<0.001$). Comparison of both the methods by the Deming regression showed that intercept and slope of the regression line were not significantly different from 0 and 1 respectively. Mean $\pm$ s.d. 3DG concentrations obtained by LC–MS/MS and LC-fluorescence were 188 ± 72 and 181 ± 61 nM respectively. Values obtained by both the methods were not significantly different. Intra- and inter-assay coefficients of variation were $<10\%$.

PG (at study visits: venous samples; home-monitored: fresh capillary blood samples) was measured with the glucose-oxidase biosensor method (EuroFlash hand-device, LifeScan, Milpitas, CA, USA; calibrated for PG with the YSI Model 2300 Glucose Analyser, YSI, Inc., Yellow Springs, OH, USA). The coefficient of variation between test strips was $<10\%$. HbA_{1c} was measured by ion-exchange HPLC method traceable to the Diabetes Control and Complication Trial (DCCT) standard (Bio-Rad VARIANT method, Bio-Rad Diagnostics Group). Amadori albumin was measured in sodium citrate plasma with ELISA (17).

N^{ϵ} -(carboxymethyl)lysine (CML) and N^{ϵ} -(carboxyethyl)lysine (CEL) residues in plasma proteins were measured by stable isotope dilution tandem MS (18).

The concentration of pyrraline in the protein-free plasma fraction was determined by UPLC–tandem MS. Because a stable isotope pyrraline internal standard was not commercially available, we have chosen, after extensive validation, [$^{13}\text{C}_9$]-3-nitrotyrosine as a nonanalog internal standard. A 25 μl plasma fraction

was mixed thoroughly with 12.5 µl internal standard (3.9 µM [$^{13}\text{C}_9$]-3-nitrotyrosine) and 500 µl methanol:acetonitrile (1:3, by volume). Proteins were removed by centrifugation (20 min at 14 000 g) and the supernatant was evaporated to dryness at 65 °C under a constant stream of nitrogen. The residue was dissolved in 150 µl water. Reversed-phase chromatography was carried out using an Acquity UPLC BEH C_{18} column (2.1 × 50 mm, 1.7 µm particle size, Waters) with 10 mM formic acid as solvent A and acetonitrile as solvent B. A linear gradient was started at 99% solvent A, which was changed in 1.5 min to 95.5% solvent A. After cleaning the column with 45% solvent B during 4 min the column was equilibrated for 2 min at the initial conditions. Injection volume was 2 µl and column temperature was set at 48 °C. Detection was carried out using a Xevo TQ tandem mass spectrometer (Waters) in electrospray-positive mode. Multiple reaction monitoring transitions were set at 236.10–189.15 for [$^{13}\text{C}_9$]-3-nitrotyrosine and at 255.10–175.20 for pyrroline. Capillary voltage was set at 0.5 kV and a cone voltage of 14 and 10 V was used for [$^{13}\text{C}_9$]-3-nitrotyrosine and pyrroline respectively. We tested recovery (and thus matrix effects), based on additions of pyrroline standard to different matrices. Mean recovery, tested in three different matrices, was $98.5 \pm 5.3\%$. Linearity was tested in 11 different matrices by adding known pyrroline concentrations between 0 and 300 nM. Mean response factor was 1.44 ± 0.11 and 1.33 ± 0.06 in plasma and water respectively. The intra-assay variation was 3.5% as determined in a plasma sample ($n=10$) with a mean pyrroline concentration of 89.8 nM. The inter-assay variation was 4.9% as determined in a plasma sample ($n=7$) with a mean pyrroline concentration of 85.2 nM. The lower limit of quantification was 19.5 fmol at an injection volume of 5 µl (signal to noise ratio (s/N)=6).

For the evaluation of endothelial dysfunction, we measured plasma levels of plasminogen activator inhibitor-1 (PAI1 or SERPINE) antigen (PAI1-ag), tissue-type plasminogen activator antigen (tPA-ag; TintElize PAI-1 and Immulyse tPA respectively; Biopool, Umeå, Sweden), and von Willebrand factor (vWf) antigen using a highly sensitive immunoassay (19). Serum levels of soluble intercellular adhesion molecule-1 (sICAM1), soluble vascular cell adhesion molecule-1 (sVCAM1), and soluble E-selectin (selectin E; sSELE) were measured using commercially available ELISA kits (Diacclone, Besançon, France).

For the evaluation of low-grade inflammation, we measured the levels of serum C-reactive protein (CRP) using a highly sensitive enzyme immunoassay (19). Plasma levels of tumor necrosis factor- α (TNF- α) and serum levels of interleukin 6 (IL6) were measured using commercially available ELISA kits (Quantikine High Sensitivity; R&D Systems, Oxon, UK) and fibrinogen was measured using the MultifibrenU reagent (Dade Behring, Marburg, Germany).

Statistical analysis

Outcome variables were evaluated after the run-in period (i.e. before initiating treatment in the first period), referred to as first-period baseline; after the washout period (i.e. before initiating treatment in the second period), referred to as second-period baseline; and on the last day of each treatment period. Treatment effects were estimated as change from first-period baseline.

Comparisons between first- and second-period baseline levels of the metabolic variables examined in this study were performed with the use of Student's *t*-test. We used a linear mixed model, with subject as a random effect, to compare the treatment effects of metformin versus repaglinide on changes in 3DG levels. Treatment type (metformin or repaglinide), treatment sequence (metformin followed by repaglinide or vice versa), the period effect, and the first-period baseline levels of 3DG were included as fixed effects. The extent to which the potential occurrence of unexpected carryover effects might have influenced sequence and period effects was thereby taken into account. Furthermore, analyses with a linear mixed model enabled information from incomplete blocks (i.e. those with dropouts) to be included (i.e. both within- and between-subject information was included).

Associations of changes in other metabolic variables with changes in 3DG were investigated by adding baseline and changes in glycemic variables (i.e. fasting (FPG), seven-point mean (MPG), AUC-PG, HbA1c, and Amadori albumin) to our initial model. Associations between changes in 3DG and changes in biomarkers of advanced glycation (i.e. CML, CEL, and pyrroline), endothelial dysfunction (i.e. PAI1-ag, tPA-ag, vWf, sICAM1, sVCAM1, and sSELE), and low-grade inflammation (i.e. CRP, TNF- α , IL6, and fibrinogen) were evaluated in a similar way. Thereby, associations of changes in these variables with changes in 3DG levels were thus adjusted for treatment type and sequence, the period effect, and first-period baseline levels of both 3DG and the other studied metabolic variable. In order to enable comparison of the strength of these associations, we used standardized (i.e. (subjects' value – population mean)/s.d.) values of changes in the metabolic variables.

Variables with skewed distribution (i.e. 3DG, pyrroline, PAI1-ag, sICAM1, sVCAM1, sSELE, CRP, TNF- α , and IL6) were logarithmically transformed prior to analyses and are presented as median and interquartile range (IQR). Treatment effects on 3DG and associations between changes in other metabolic variables and changes in 3DG and between changes in 3DG and changes in biomarkers with skewed distribution are presented as percentages due to required back transformation of the regression coefficients.

All statistical analyses were carried out using the Statistical Package for Social Sciences, version 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

Patients' characteristics at enrollment are shown in Table 1. A more detailed description of patients' characteristics (e.g. use of medication) at enrollment as well as of adverse events during the trial have been presented elsewhere (14).

Table 2 shows first- (i.e. after 1-month run-in on diet-only treatment) and second-period (i.e. after 1-month washout on diet-only treatment) baseline data of 3DG and the other metabolic variables examined, according to each treatment sequence. No significant differences were found between first- and second-period baseline data.

Effects of metformin or repaglinide treatment on 3DG

3DG levels decreased after both metformin (-19.3% (95% confidence interval (CI): -23.5 , -14.8)) and repaglinide (-20.8% (95% CI: -24.9 , -16.3)) treatments (Fig. 2), but no significant difference was found between the two treatments (1.8% (95% CI: -3.8 , 7.8)).

Table 1 Clinical characteristics of the study participants at enrollment. Data are expressed as mean $\pm$ s.d., median (range) or percentages. Data are stratified according to the treatment sequence: repaglinide followed by metformin or vice versa.

	Repaglinide followed by metformin (n=48)	Metformin followed by repaglinide (n=48)
Men/women (n)	36/12	37/11
Age (years)	63.3 $\pm$ 8.9	59.5 $\pm$ 9.3
Known duration of diabetes (years)	5 (0–28)	3 (0–22)
Body mass index (kg/m ²)	24.8 $\pm$ 1.9	24.7 $\pm$ 2.1
HbA1c (%)	7.6 $\pm$ 0.9	7.3 $\pm$ 0.8
Smoking, n (%)	28 (58)	27 (56)
Late diabetic complications (%) ^a		
Retinopathy (none/simplex/proliferative)	65/33/2	73/21/6
Macroangiopathy	21	23
Nephropathy (normo-/micro-/macroalbuminuria)	71/25/4	73/23/4
Neuropathy	75	71
Prestudy anti-hyperglycaemic treatment (%)		
Diet-only	19	15
Metformin	13	8
Repaglinide	8	0
Sulfonylurea	44	63
Metformin plus sulfonylurea	17	15
Ongoing nonstudy medication (%)		
Antihypertensive medication	63	46
Lipid-lowering medication	35	25
Aspirin	44	46

^aNote that the proportion of patients with diabetic complications have been changed (13, 15) from the original publication (14) after checking clinical signs with patients' medical records.

Associations between changes in glycemic variables and changes in 3DG

To explore a potential pathway through which both treatments could have induced decreases in 3DG levels, we investigated the associations between changes in a range of glycemic variables and changes in 3DG.

Changes in all glycemic variables were positively associated with changes in 3DG (Table 3). Specifically, for each s.d. decrease in FPG, 3DG decreased by 22.7% (95% CI: 19.0, 26.5). Similarly, 3DG decreased by 20.0% (95% CI: 16.2, 23.9) per s.d. decrease in seven-point MPG, by 22.5% (95% CI: 18.6, 26.6) per s.d. decrease in AUC-PG, and by 17.2% (95% CI: 13.8, 20.6) per s.d. decrease in HbA1c. Noticeably, the magnitudes of these associations were similar. In addition, for each s.d. decrease in Amadori albumin, 3DG decreased by 10.9% (95% CI: 6.4, 15.5).

Associations between changes in 3DG and changes in biomarkers of advanced glycation, endothelial dysfunction, and inflammation

To explore potential pathways through which decreases in 3DG levels could influence the cardiovascular risk profile, we investigated the associations between changes in 3DG and changes in biomarkers of advanced glycation, endothelial dysfunction, and inflammation.

For each s.d. decrease in 3DG, CML decreased by 0.17 s.d. (95% CI: 0.01, 0.33), but no associations were found between changes in 3DG and changes in CEL (0.08 s.d. (95% CI: -0.07 , 0.24)) or pyrraline (-1.8% (95% CI: -11.4 , 8.8)) (Table 4).

Furthermore, the treatment-induced reduction in 3DG was associated with a reduction in the endothelial markers PAI-1-ag, tPA-ag, sVCAM-1, sSELE, but not with changes in vWf and sICAM-1. In addition, we found that treatment-induced reduction in 3DG was not associated with statistically significant reductions in the inflammatory markers TNF- α , CRP, IL6, and fibrinogen (Table 5).

Additional analyses

Additional adjustment for changes in body mass, which differed between treatments (14) and may affect the metabolic variables examined herein, did not substantially influence our results. Specifically, after the additional adjustment for changes in body mass, 3DG levels decreased after both metformin (-18.0% (95% CI: -22.4 , -13.6)) and repaglinide (-22.0% (95% CI: -26.1 , -17.7)) treatments, but no significant difference was found between the two treatments (-4.8% (95% CI: -10.4 , 1.1)). In addition, adjustment for changes in body mass did not substantially influence the associations between changes in glycemic variables and biomarkers of advanced glycation, endothelial dysfunction, and low-grade inflammation and changes in 3DG (data not shown).

Table 2 First- and second-period baseline values of the studied metabolic variables. Data are expressed as mean $\pm$ s.d. or median (interquartile range), and stratified according to the treatment sequence: repaglinide followed by metformin (RE–MET) or vice versa (MET–RE).

	First-period baseline		Second-period baseline		P value	
	RE–MET (n=48)	MET–RE (n=48)	RE–MET (n=45)	MET–RE (n=41)	RE–MET	MET–RE
3-Deoxyglucosone (nM)	1164 (945; 1574)	1185 (865; 1487)	1122 (854; 1648)	1089 (903; 1403)	0.855	0.539
FPG (mmol/l)	14.0 $\pm$ 4.0	12.8 $\pm$ 3.5	14.4 $\pm$ 4.4	13.6 $\pm$ 4.2	0.344	0.074
Seven-point MPG (mmol/l) ^a	13.0 $\pm$ 4.4	12.2 $\pm$ 3.1	13.6 $\pm$ 4.0	11.5 $\pm$ 3.2	0.208	0.121
AUC-PG (time ^a mmol/l) ^b	6008 $\pm$ 1652	5498 $\pm$ 1528	6083 $\pm$ 1760	5630 $\pm$ 1658	0.692	0.438
HbA1c (%)	8.2 $\pm$ 1.2	7.8 $\pm$ 0.8	8.0 $\pm$ 1.5	7.8 $\pm$ 1.2	0.246	0.656
Amadori albumin (U/ml)	50.2 $\pm$ 11.1	48.7 $\pm$ 12.2	48.6 $\pm$ 12.4	48.9 $\pm$ 10.9	0.346	0.873
CML (mmol/mol lysine)	0.069 $\pm$ 0.015	0.068 $\pm$ 0.015	0.070 $\pm$ 0.012	0.070 $\pm$ 0.015	0.515	0.064
CEL (mmol/mol lysine)	0.016 $\pm$ 0.003	0.016 $\pm$ 0.003	0.016 $\pm$ 0.003	0.016 $\pm$ 0.003	0.713	0.845
Pyrraline (nM)	17.4 (9.6; 26.3)	17.6 (11.6; 26.0)	20.3 (13.0; 27.1)	17.4 (10.6; 25.6)	0.104	0.993

AUC, area under the curve during the 6 h meal test; CML, N^ε-(carboxymethyl)lysine; CEL, N^ε-(carboxyethyl)lysine.

^aIn the RE–MET group, n=36 and n=32 and in the MET–RE group n=37 and n=24 for the first- and second-period baseline respectively.

^bIn the RE–MET group, n=46 and n=42 and in the MET–RE group n=45 and n=38 for the first- and second-period baseline respectively.

Discussion

The present single-center, randomized, double-masked, double-dummy, crossover study of 96 nonobese insulin-naïve individuals with type 2 diabetes comparing the effects of metformin and repaglinide treatment on blood levels of 3DG had three main findings. First, 3DG levels decreased to the same extent either after treatment with metformin or with repaglinide. Secondly, changes in several glycemic variables, i.e. FPG, seven-point MPG, AUC-PG, HbA1c, and Amadori albumin, were strongly and positively associated with these changes in 3DG levels. Thirdly, metformin- and repaglinide-induced changes in 3DG were positively associated with changes in CML (i.e. an AGE that can be formed by 3DG) and several markers of endothelial dysfunction (i.e. PAI1, tPA-ag, sVCAM1, and sSELE).

Given the similarities in structure between metformin and aminoguanidine, it has been proposed that metformin reacts with the dicarbonyl groups of

α -oxoaldehydes in a similar way to aminoguanidine, thereby preventing the toxicity of these α -oxoaldehydes and the subsequent production of AGEs (9–11). In this study, we indeed found a decrease in 3DG levels after treatment with metformin, which is in agreement with earlier observations of lower levels of α -oxoaldehydes and AGEs in patients with type 2 diabetes treated with this drug (9, 11). Remarkably, we found a similar decrease in 3DG levels after treatment with another antihyperglycemic agent, repaglinide, which is a short-acting insulin secretagogue belonging to the meglitinide analogues.

The different working mechanisms of the antihyperglycemic effects of metformin and repaglinide suggest that improvement in glycemic control, rather than a specific effect of metformin on quenching of 3DG, results in a decrease in 3DG. This is concordant with a similar HbA1c-lowering effect of either treatment as previously reported (14) and further supported by our current findings, showing that changes in 3DG were strongly associated with changes in several glycemic variables, independently of treatment type, treatment sequence, and period effect.

Both metformin (12, 19) and repaglinide (20, 21) have been described to hold cardiovascular potentials in patients with type 2 diabetes, though a greater beneficial role has been attributed to metformin, which has also been previously shown in this study (15). Our current findings showing a similar beneficial effect of both drugs on 3DG levels, which are associated with diabetic microangiopathy (5–7), suggest that the metformin- or repaglinide-induced reduction in 3DG may explain part of the beneficial effect of both drugs on the cardiovascular risk profile.

We have also found a strong association between changes in AUC-PG and changes in 3DG. Postprandial rise in 3DG (6) and the association between postprandial glucose levels and levels of α -oxoaldehydes including 3DG have been described previously (22).

Figure 2 Levels of 3DG at baseline and after treatment with metformin or repaglinide. Boxes indicate the median values and the interquartile ranges and the whiskers represent the minimum and maximum values. P values for differences between baseline and after 4 months of treatment were calculated using a linear mixed model (see 'Materials and methods' section).

Table 3 Associations of changes in glycemic variables with percent changes in 3DG. The regression coefficient β represents the percent change (i.e. decrease) in 3DG levels with a change (i.e. decrease) of 1 s.d. in the studied glycemic variables. All analyses were adjusted for treatment type, treatment sequence, the period effect, and baseline levels of 3DG and the studied glycemic variables.

	Δ 3DG		
	β	95% CI	P value
Δ FPG	22.7	19.0; 26.5	<0.001
Δ Seven-point MPG	20.0	16.2; 23.9	<0.001
Δ AUC-PG	22.5	18.6; 26.6	<0.001
Δ HbA1c	17.2	13.8; 20.6	<0.001
Δ Amadori albumin	10.9	6.4; 15.5	<0.001

CI, confidence interval; AUC, area under the curve during 6 h meal test.

In this regard it is important to emphasize that postprandial fluctuations in glucose levels have been thought to be more strongly associated with complications than high-glucose levels in general (23, 24). Although the exact mechanisms explaining the increased risk for cardiovascular disease due to glucose fluctuations are unknown, increased oxidative stress by glucose fluctuations during postprandial periods seems to be involved (23, 24). Such increases in oxidative stress may be due to increased formation of α -oxoaldehydes (6). Therefore, both an increase in oxidative stress and dicarbonyl stress and consequent increased formation of AGEs may affect the vasculature and thus explain the increased cardiovascular risk related to postprandial fluctuations in glucose levels.

Two major pathways for the formation of 3DG have been described (25). 3DG can be formed in the polyol pathway, in which 3DG is a hydrolysis product of fructose-3-phosphate, and through the Maillard reaction. In the latter, the nonenzymatic glycation of protein amino groups leads to the formation of an Amadori product, which can then undergo multiple dehydration reactions and rearrangements to produce highly reactive carbonyl compounds such as 3DG. In accordance, we found that levels of Amadori albumin decreased after antihyperglycemic treatment and that this change was associated with a decrease in blood levels of 3DG. 3DG can react with free amino groups to form AGEs including CML and pyrraline (25). Indeed, our current findings show an association between changes in 3DG and changes in CML after glucose-lowering treatment. However, we could not find such an association with changes in pyrraline. These differences might be explained by the fact that CML, rather than pyrraline, is a major AGE structure in 3DG-modified proteins (26).

Endothelial dysfunction and low-grade inflammation have been described as potential pathways to explain the increased risk for cardiovascular disease in both type 1 (27, 28) and type 2 diabetes (29) and the increased formation of AGEs could have an important role in these

pathways (30, 31). Indeed, our current results show an association between a glucose-lowering treatment-induced reduction in 3DG and a reduction in markers of endothelial dysfunction – but not of low-grade inflammation – which further supports the view that this could constitute a pathway through which inhibition of the glycation pathway may have a beneficial impact on the cardiovascular risk profile. Moreover, from this study, we previously have shown that metformin had a more pronounced effect to lower such markers of endothelial dysfunction than did repaglinide despite overall similar glycemic control (i.e. independent of glycemic control) (15). Thus, together with our present data indicating equal potency of metformin and repaglinide to lower 3DG as well as an association between 3DG and markers of endothelial dysfunction, this suggests that metformin might have a potential beneficial influence on such markers of cardiovascular risk through both shared (glycemic) and specific (nonglycemic) pathways.

Some limitations to this study need to be mentioned. First, the lack of a 'nontreatment' or 'diet-only' arm does not enable us a full understanding of the importance of magnitude of the effects observed on 3DG levels after treatment with both metformin and repaglinide. Second, the study was not designed to assess the exact mediating effects of treatment-derived changes in the different glycemic variables on changes in 3DG. However, the strong and consistent associations between changes in the different variables – regardless of treatment type – and the fact that treatment has a similar effect on both 3DG and other glycemic variables suggest that changes in other glycemic variables, such as PG levels and HbA1c, at least partially explained the reduction in 3DG induced by metformin and repaglinide. Third, the presence of carryover effects in a trial with a crossover design could constitute such another limitation. However, the two treatment sequence groups (i.e. metformin followed by repaglinide or vice versa) were well balanced and comparable at each baseline measurement, and a possible carryover effect was adjusted for to the extent it influenced the effect of

Table 4 Associations of changes in 3DG with changes in biomarkers of advanced glycation. The regression coefficient β represents the changes (in s.d. for CML and CEL; in % for pyrraline) in the studied biomarker of advanced glycation with a change of 1 s.d. in 3DG levels. All analyses were adjusted for treatment type, treatment sequence, the period effect, and baseline levels of 3DG and the studied biomarker of advanced glycation.

	Δ 3DG		
	β	95% CI	P value
Δ N ^ε -CML	0.17	0.01; 0.33	0.044
Δ N ^ε -CEL	0.08	−0.07; 0.24	0.286
Δ Pyrraline	−1.8	−11.4; 8.8	0.724

CI, confidence interval.

Table 5 Associations of changes in 3DG with changes in biomarkers of endothelial dysfunction and low-grade inflammation. The regression coefficient β represents the changes (in s.d. for tPA-antigen, vWf, fibrinogen; in % for PAI1, sICAM, sVCAM, sSELE, CRP, TNF- α , IL6) in the studied biomarker of endothelial dysfunction or low-grade inflammation with a change of 1 s.d. in 3DG levels. All analyses were adjusted for treatment type, treatment sequence, the period effect, and baseline levels of 3DG and the studied biomarker of endothelial dysfunction or low-grade inflammation.

	Δ 3DG		
	β	95% CI	P value
Endothelial dysfunction			
Δ PAI1	19.1	1.3; 40.0	0.034
Δ tPA-ag	0.17	0.02; 0.33	0.029
Δ vWf	0.08	-0.10; 0.25	0.383
Δ sICAM1	3.9	-12.3; 23.0	0.658
Δ sVCAM1	20.5	1.5; 43.1	0.034
Δ sSELE	19.1	0.5; 41.2	0.044
Low-grade inflammation			
Δ CRP	0.8	-16.7; 18.2	0.929
Δ TNF- α	16.4	-1.0; 36.8	0.065
Δ IL6	-5.3	-19.4; 11.2	0.501
Δ Fibrinogen	-0.03	-0.19; 0.14	0.751

CI, confidence interval.

period and treatment sequence. We have analyzed the between-treatment effects as changes from first-period baseline. We choose this because the second-period baseline could be biased between the treatment sequences by possible differential carryover effects arising from the two treatments being stopped only 1 month earlier when completing the first-treatment period. In contrast, we did not have a specific reason to suspect such potential differential effects from previous glucose-lowering treatments at the first-period baseline (because previous such therapies were equally distributed between the treatment sequences (Table 1); $P > 0.1$). However, additionally using the second-period baseline in the analyses did not substantially influence our results (data not shown). Finally, this trial was carried out with nonobese patients with type 2 diabetes. Therefore, the extent to which our findings may be extrapolated to obese patients remains uncertain. Of note is that more than 20% of Caucasian patients with type 2 diabetes are nonobese (32). In these patients and in contrast to those who are obese, solid scientific evidence supporting metformin as 'drug of first choice', as opposed to an insulin secretagogue, is however still lacking.

In conclusion, the improved glycemic control in type 2 diabetes induced by metformin and repaglinide is associated with a reduction in 3DG levels, which is similar in magnitude for both drugs and associated with reductions in AGEs and endothelial markers. This may constitute a metabolic pathway through which both treatments have a beneficial impact on the cardiovascular risk profile.

Declaration of interest

S S Lund, L Tarnow, H-H Parving, and A Vaag have reported equity in Novo Nordisk A/S. L Tarnow, O Pedersen, H-H Parving, and A Vaag have received funds from Novo Nordisk A/S for the research. S S Lund and A Vaag have received fees from Novo Nordisk A/S for speaking and A Vaag has received fees from Novo Nordisk A/S for organizing education. S S Lund, L Tarnow, H-H Parving, O Pedersen, and A Vaag are present or former employees of Steno Diabetes Center, Gentofte, Denmark. Steno Diabetes Center is an independent academic institution owned by Novo Nordisk A/S and The Novo Nordisk Foundation. The sponsors had no role in the study design, collection, analysis, or interpretation of data, writing the report, or the decision to submit the paper for publication.

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