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ORIGINAL ARTICLE

Relationships between glucose excursion and the activation of oxidative stress in patients with newly diagnosed type 2 diabetes or impaired glucose regulation

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Abstract The effect of glucose excursions on oxidative stress is an important topic in diabetes research. We investigated this relationship by analyzing markers of oxidative stress and glycemic data from a continuous glucose monitoring system (CGMS) in 30 individuals with normal glucose regulation (NGR), 27 subjects with impaired glucose regulation (IGR), and 27 patients with newly diagnosed type 2 diabetes (T2DM). We compared the mean amplitude of glycemic excursion (MAGE), mean postprandial glucose excursion (MPPGE), and mean postprandial incremental area under the curve (IAUC) with plasma levels of oxidative stress markers 8-iso-PGF2α, 8-OH-dG, and protein carbonyl content in the study subjects. Patients with T2DM or IGR had significantly higher glucose excursions and plasma levels of oxidative stress markers compared to normal controls ($P < 0.01$ or 0.05). Multiple linear regression analyses showed significant relationships between MAGE and plasma 8-iso-PGF2α, and between MPPGE and plasma 8-OH-dG in patients with IGR or T2DM ($P < 0.01$ or 0.05). Furthermore, 2h-postprandial glucose level and IAUC were related to plasma protein carbonyl content in the study cohort including T2DM and IGR ($P < 0.01$). We demonstrate that glucose excursions in subjects with IGR and T2DM trigger the activation of oxidative stress.

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Introduction

Diabetes is characterized by the development of specific micro- and macro-vascular complications. Large-scale, prospective studies such as the Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS) have shown that diabetic complication is at least partially dependent upon hyperglycemia [1, 2]. A theory linking hyperglycemia and diabetic complications was developed by Brownlee, which suggests that hyperglycemia-induced overproduction of superoxide plays a critical role in the biochemical abnormalities leading to vascular disease and diabetic complications [3]. The condition of chronic hyperglycemia can present in two different forms: sustained hyperglycemia and fluctuating hyperglycemia [4, 5]. Glycated hemoglobin (HbA1c), an indicator of chronic sustained hyperglycemia, is the standard by which glycemic control is measured. However, the Epidemiology of Diabetes Interventions and Complications (EDIC) study showed that conventionally treated subjects experienced a higher risk of progression to diabetic retinopathy than the intensively treated group, even in cases of comparable HbA1c levels [6]. The researchers therefore suggested that glucose excursion is of great importance in the development of diabetic vascular complications [7, 8].

Studies in vitro have shown that conditions of intermittent high glucose lead to vascular endothelial cell apoptosis through mitochondrial superoxide overproduction [9, 10]. In vivo studies of this nature have only recently been made available by the development of continuous glucose monitoring system (CGMS), which allows

complete and precise assessment of glucose fluctuations in vivo [11]. In order to identify the occurrence of superoxide production, various markers of oxidative damage have been identified, including 8-iso-prostaglandin F2α (8-iso-PGF2α) as a marker of lipid peroxidation, protein carbonyl content as a marker of oxidative modification of proteins, and 8-hydroxydeoxyguanosine (8-OH-dG) as a sensitive indicator of oxidative damage to DNA in vivo [12–14].

Monnier et al. reported that 24 h urinary excretion rates of 8-iso-PGF2α in 21 type 2 diabetic patients was significantly related to the mean amplitude of glycemic excursions (MAGE), calculated from CGMS data. Furthermore, they found glucose fluctuations exhibited a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia estimated from HbA1c [15]. The objective of our study was to help clarify the effect of glucose excursions on activation of oxidative stress using CGMS data collected from subjects with normal glucose regulation (NGR), patients with impaired glucose regulation (IGR), and patients with newly diagnosed type 2 diabetes (T2DM).

Results

Glucose excursions in subjects with varying glucose regulation

Physical and clinical characteristics of subjects with NGR ($n = 30$), subjects with IGR ($n = 27$), and those with T2DM ($n = 27$) are shown in Table 1. Blood glucose levels of all study subjects were continuously monitored over a 3-day period using CGMS. The mean of the three daily values for each time point were plotted to show the pattern of glucose excursion over 24 h for subjects in each category of glucose regulation (Fig. 1). Using the CGMS data, glucose excursion was assessed using several parameters measuring intra- and inter-day glycemic variabilities. All measurements of glucose level and excursion (MBG, MAGE, MPPGE, MODD, and IAUC) were highest in patients with newly diagnosed T2DM, decreased in subjects with IGR, and lowest in those with NGR (Table 2). After adjustment for age, BMI, waist circumference, systolic blood pressure (SBP), and triglycerides (TG), the differences in these values between T2DM and NGR were found to be statistically significant ($P < 0.01$). These values were also statistically significantly higher in T2DM than in subjects with IGR ($P < 0.01$ or 0.05), with the exception of IAUC, in which no significant difference was found between T2DM and IGR (Table 2). Additionally, all the measures of glucose excursion were statistically significantly higher in patients with IGR compared to

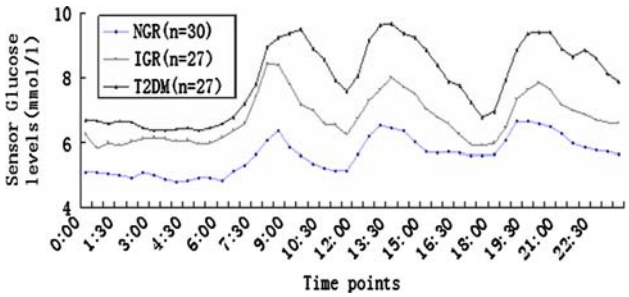

Fig. 1 Twenty-four hour glucose profile in subjects with different glucose regulation. *NGR* normal glucose regulation, *IGR* impaired glucose regulation, *T2DM* type 2 diabetes

Table 1 Clinical characteristics of the study participants

Parameters	NGR ($n = 30$)	IGR ($n = 27$)	T2DM ($n = 27$)	P
Age (years)	48.5 ± 14.7	54.1 ± 14.7	47.7 ± 11.4	0.12[a]
No. of men/women	15/15	15/12	16/11	0.778[b]
BMI (kg/m^2)	21.7 ± 2.00	23.4 ± 2.72	25.2 ± 2.54	<0.01[a]
Waist circumference (cm)	78.7 ± 4.92	83.0 ± 7.94	87.3 ± 8.31	<0.01[a]
Blood pressure (mmHg)				
Systolic	115.3 ± 11.2	134.7 ± 18.6	130.7 ± 18.6	<0.01[a]
Distolic	75.2 ± 6.5	80.9 ± 9.5	82.0 ± 10.9	0.013[a]
HbA1c (%)	ND	5.88 ± 0.59	6.51 ± 0.96	<0.01[c]
Total cholesterol (mmol/l)	4.30 ± 0.67	4.68 ± 1.20	4.59 ± 1.13	0.343[a]
Triglycerides (mmol/l)	1.30 ± 0.36	1.74 ± 0.79	2.19 ± 1.21	<0.01[a]
HDL cholesterol (mmol/l)	1.52 ± 0.39	1.28 ± 0.36	1.20 ± 0.41	<0.01[a]
LDL cholesterol (mmol/l)	2.28 ± 0.61	2.56 ± 1.07	2.32 ± 1.01	0.460[a]
Serum creatinine (μmol/l)	71.6 ± 14.4	73.1 ± 17.9	67.6 ± 12.7	0.395[a]
Fasting plasma glucose (FPG) (mmol/l)	5.23 ± 0.27	6.27 ± 0.47	7.54 ± 1.62	<0.01[a]
2h postprandial plasma glucose(2h PG) (mmol/l)	5.70 ± 1.14	8.13 ± 1.65	14.78 ± 3.82	<0.01[a]

Values given as number or mean ± SD

HbA1c hemoglobin A1c, *2h PG* 2h postprandial glucose level in OGTT

[a] Statistical differences between groups tested using one-way ANOVA

[b] Statistical differences between groups tested using chi-square test

[c] Statistical differences between groups tested using unpaired Student's *t* test

Table 2 Parameters measuring glucose excursion and the activation of oxidative stress (italicized) in subjects with varying glucose regulation over 3 days using CGMS

Parameters	NGR ($n = 30$)	IGR ($n = 27$)	T2DM ($n = 27$)
MBG (mmol/l)	5.67 ± 0.52	6.76 ± 0.62**	8.09 ± 1.84**##
MAGE (mmol/l)	1.60 ± 0.79	2.49 ± 1.16**	3.46 ± 1.51**##
MODD (mmol/l)	0.68 ± 0.27	1.07 ± 0.46**	1.34 ± 0.43**#
MPPGE (mmol/l)	2.03 ± 0.93	3.04 ± 1.44**	4.12 ± 1.57**##
IAUC (mmol l^{-1} h^{-1})	2.85 ± 1.90	5.29 ± 3.42**	6.08 ± 4.18**
8-iso-PGF2α (pg/ml)	*155.80 (40.40)*	*198.63 (88.04)**	*277.56 (202.08)**#
8-OH-dG (ng/ml)	*2.48 (2.30)*	*4.71 (2.76)***	*5.96 (5.38)**##
Protein carbonyl (nmol/ml)	*26.91 ± 10.05*	*83.95 ± 29.09***	*104.97 ± 38.24**#▲

Values of glucose excursion parameters are the mean ± SD of data collected for the 3-day study period. Skewed variables of oxidative stress (plasma 8-iso-PGF2α and 8-OH-dG) were presented as median and quartile range M (QR) and were ln-transformed to give normal distributions. Linear regression models in multivariate analyses were used to adjust for the potential confounders such as BMI, waist circumference, and HbA1c

MBG mean blood glucose over a 24-h period, *MAGE* mean intra-daily amplitude of glycemic excursion, *MODD* mean of inter-daily differences, *MPPGE* mean postprandial glucose excursion over a 24-h period, *IAUC* mean postprandial incremental area under the curve over a 24-h period

* $P < 0.05$ and ** $P < 0.01$ compared to subjects with NGR

# $P < 0.05$ and ## $P < 0.01$ compared to subjects with IGR

▲ Statistically significant difference between T2DM and IGR removed after adjustment for HbA1c

NGR ($P < 0.01$), after adjustment for age, BMI, waist circumference, SBP, and TG (Table 2).

Activation of oxidative stress in subjects under different glucose regulation

Vascular endothelial cell apoptosis mediated by mitochondrial superoxide overproduction may be brought on by intermittent high glucose. We decided to test whether levels of three indicators of oxidative stress correlated with glucose excursions in the test subjects. Markers of lipid peroxidation (8-iso-PGF2α), oxidative modification of proteins (protein carbonyl content), and a sensitive indicator of oxidative damage to DNA in vivo (8-OH-dG) were measured. We observed that 24 h urinary levels of 8-iso-PGF2α and 8-OH-dG were well correlated with the corresponding plasma levels 3 h after breakfast when corrected for creatinine concentration in 10 patients for whom urinary samples were obtained. Urinary protein carbonyl content was not assayed due to low protein concentration (data not shown). We therefore measured the levels of oxidative stress indicators in plasma samples of all study subjects. Plasma levels of 8-iso-PGF2α, 8-OH-dG, and protein carbonyl were highest in patients with newly diagnosed T2DM, intermediate in subjects with IGR, and lowest in those with NGR after adjustment for age, BMI, waist circumference, SBP, and TG (Table 2, italics). These differences were statistically significant between T2DM and NGR ($P < 0.01$), as well as between IGR and NGR ($P < 0.01$ or 0.05). However, the difference in protein carbonyl levels between T2DM and IGR became insignificant when further adjusted for HbA1c (Table 2, italics).

Relationship between activation of oxidative stress and glucose excursions

Multiple linear stepwise regression analyses were used to assess the effects of different variables, including BMI, waist circumference, SBP, DBP, triglycerides, total cholesterol, high- (HDL) and low-density lipoprotein (LDL) cholesterol concentrations, fasting plasma glucose (FPG), and 2h-postprandial glucose (2hPG) level, as well as parameters of glucose excursions on the activation of oxidative stress.

We found that plasma 8-iso-PGF2α concentration was dependent on the variables MBG, MAGE, and IAUC in subjects with IGR, and on variables HbA1c, MAGE, and IAUC in patients with T2DM in multiple linear regression analyses. Parameters of MAGE and IAUC were found to be highly correlated in a study cohort including subjects with T2DM and IGR ($r = 0.776$, $P < 0.001$), we therefore used separate models in multiple linear regression analyses. Entering MBG and MAGE into the model resulted in an adjusted coefficient of determination (R^2) of 0.370 ($P = 0.001$) in subjects with IGR, and the standard regression coefficient for MAGE was 0.508 ($P < 0.01$) (Table 3, top, IGR model a). Similarly, entering HbA1C and MAGE into the model, we found the adjusted R^2 to be 0.308 ($P < 0.01$) in patients with T2DM, and the standard regression coefficient for MAGE was 0.381 ($P < 0.05$)

Table 3 Effects of markers of diabetic control on the activation of oxidative stress by model

Study participants	Models	Markers	β	t	P	Adjusted R^2 of the model
ln Plasma 8-iso-PGF2α						
IGR	Model a	MBG	0.234	1.350	0.190	
		MAGE	0.508	2.923	0.007	0.370
	Model b	*MBG*	*0.381*	*2.248*	*0.034*	
		IAUC	*0.369*	*2.178*	*0.039*	*0.287*
T2DM	Model a	HbA1c	0.360	2.048	0.052	
		MAGE	0.381	2.167	0.041	0.308
	Model b	*HbA1c*	*0.392*	*2.182*	*0.04*	
		IAUC	*0.311*	*1.731*	*0.097*	*0.263*
ln Plasma 8-OH-dG						
IGR	Model	MBG	0.268	1.435	0.164	
		MPPGE	0.437	2.335	0.028	0.332
T2DM	Model	FPG	0.247	1.424	0.168	
		MPPGE	0.481	2.773	0.011	0.291
Plasma protein carbonyl						
IGR and T2DM	Model	Waist circumference	0.153	1.447	0.155	
		HbA1c	0.231	1.844	0.071	
		2hPG	0.350	2.834	0.007	
		IAUC	0.286	2.697	0.010	0.464

Highly correlated parameters of IAUC and MAGE entered the regression equation at the same time when ln-transformed plasma 8-iso-PGF2α used as a dependent variable, we therefore used separate models, a and b

IGR impaired glucose regulation, *T2DM* type 2 diabetes, *MBG* mean blood glucose, *MAGE* mean amplitude of glycemic excursion, *HbA1c* hemoglobin A1c, *MPPGE* mean postprandial glucose excursion, *IAUC* mean postprandial incremental area under the curve, *FPG* fasting plasma glucose, *2hPG* 2h postprandial glucose level in OGTT, β standard regression coefficient

(Table 3, top, T2DM model a). Performing the analysis with IAUC substituted for MAGE resulted in a decrease in the multiple R^2 of the model from 0.370 to 0.287 in patients with IGR and from 0.308 to 0.263 in patients with T2DM (Table 3, top, models b italicized). A simple linear analysis showed positive correlations between ln-transformed plasma 8-iso-PGF2α levels and MAGE in subjects with IGR ($r = 0.612$, $P = 0.001$) and in patients with newly diagnosed T2DM ($r = 0.498$, $P = 0.01$) (Fig. 2a).

Further multiple stepwise linear regression analysis showed that plasma 8-OH-dG level, another indicator of oxidative stress, was dependent on the variables MBG and MPPGE in subjects with IGR and on variables fasting plasma glucose (FPG) and MPPGE in patients with T2DM. The adjusted R^2 of the model was 0.332 ($P < 0.01$) in subjects with IGR, with a standard regression coefficient for MPPGE of 0.437 ($P < 0.05$) (Table 3, middle, IGR model). For patients with T2DM, modeling with FPG and MPPGE gave an adjusted R^2 of 0.291 ($P < 0.01$) with a standard regression coefficient for MPPGE of 0.481 ($P < 0.05$) (Table 3, middle, T2DM model). Simple linear analysis showed a positive correlation between ln-transformed plasma 8-OH-dG level and MPPGE in patients with newly diagnosed T2DM ($r = 0.539$, $P < 0.01$) or IGR ($r = 0.575$, $P < 0.01$) (Fig. 2b).

Finally, we analyzed the relationship between plasma protein carbonyl content and different variables in the study cohort including all patients with abnormal glucose metabolism (newly diagnosed T2DM and IGR, for no difference in plasma protein carbonyl content was found between the two groups after adjustment for HbA1c). In multiple linear stepwise regression analysis, we found that plasma protein carbonyl content was dependent on 2hPG, waist circumference, HbA1c, and IAUC. The adjusted R^2 of the model was 0.464 ($P < 0.001$), with a standard regression coefficient for 2hPG of 0.350 ($P < 0.01$) and for IAUC of 0.286 ($P = 0.01$) (Table 3, bottom, model). Simple linear analysis showed that plasma protein carbonyl content was positively correlated with IAUC ($r = 0.443$, $P = 0.001$; Fig. 2c).

Discussion

Activation of oxidative stress may be associated with diabetes and its chronic complications [13, 16–18]. In the

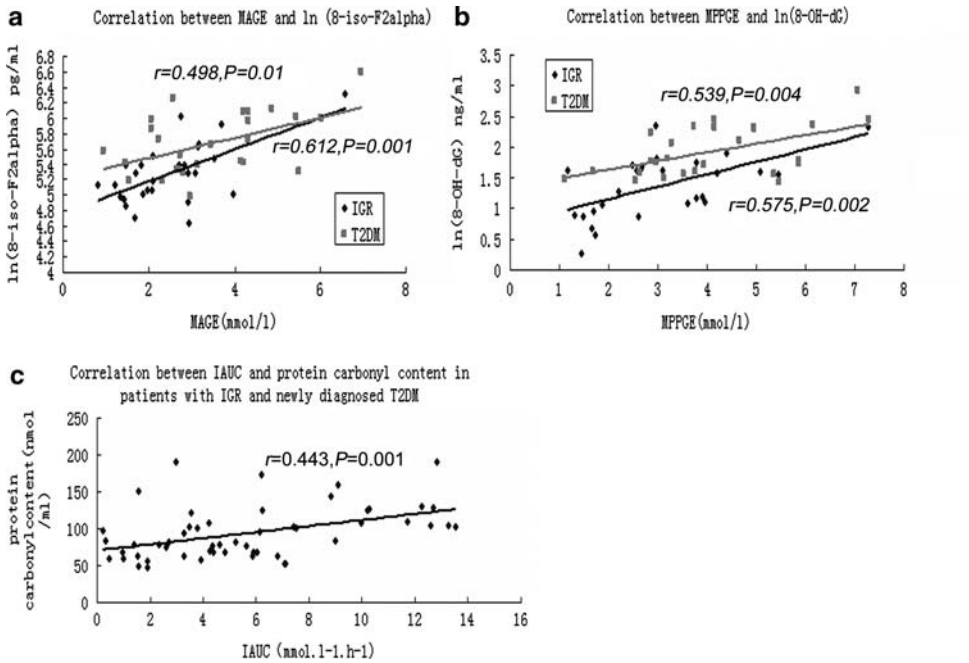

Fig. 2 Relationships between various markers of glucose excursion and the activation of oxidative stress. *IGR* impaired glucose regulation, *T2DM* type 2 diabetes, *MAGE* mean amplitude of glycemic excursion, *MPPGE* mean postprandial glucose excursion, *IAUC* mean postprandial incremental area under the curve. **a** Linear correlation between MAGE and ln-transformed 8-iso-PGF2α levels in plasma samples from patients with IGR or T2DM. **b** Linear correlation between MPPGE and ln-transformed 8-OH-dG levels in plasma samples from patients with IGR or T2DM. **c** Linear correlation between IAUC and plasma protein carbonyl content in patients with IGR and T2DM

present study, we observed evidence of increased DNA oxidative damage, and lipid and protein oxidations in diabetic patients compared to those with NGR, as shown by higher plasma 8-iso-PGF2α, 8-OH-dG, and protein carbonyl levels. Higher levels of the markers of oxidative stress were even found in patients with IGR compared to normal controls in our study. Importantly, our data suggest that the activation of oxidative stress was more dependent upon glucose excursions than chronic sustained hyperglycemia in subjects with IGR and patients with newly diagnosed T2DM.

A derivative produced during peroxidation of arachidonic acid, 8-iso-PGF2α, is used as a measure of lipid peroxidation in vivo [19]. Consistent with the findings of Monnier [15], we found that glucose fluctuation estimated by MAGE was an independent determinant of plasma 8-iso-PGF2α levels in patients with newly diagnosed T2DM and in those with IGR. Therefore, individuals with IGR, whose HbA1c levels are still near normal and are not experiencing conditions of chronic hyperglycemia, are likely experiencing increased activation of lipid oxidation due to glucose excursions. Other than MAGE, which takes into account upward and downward glycemic changes over a 24-h period, plasma 8-iso-PGF2α levels were also dependent upon IAUC, an estimate of postprandial upward glycemic changes. We also observed a decrement, but a lesser extent in R^2 of the model in patients with T2DM than that of Monnier's when we substitute IAUC for MAGE in our multivariate analysis, and the difference might be interpreted as evidence that glucose fluctuations observed in our study were mainly postprandial increases, for the enrolled subjects with T2DM were newly diagnosed and had never received anti-diabetic treatment that might induce a hypoglycemic episode, furthermore, no such episode (sensor glucose value <2.8 mmol/l) was detected by CGMS. A previous study has shown that postprandial hyperglycemia, especially in the form of hyperglycemic spikes, induces overproduction of superoxide, leading to endothelial damages [20]. However, from this study, together with Monnier's findings, we speculate that the triggering effect of glucose fluctuation on lipid oxidation is likely due to several types of glucose fluctuation including postprandial glucose increases as well as hypoglycemic episodes over inter-prandial and midnight periods, which are quite common in type 1 diabetes and to a lesser degree, in type 2 diabetes treated with insulin. Therefore, in patients with T2DM, interventions aimed at reducing vascular damage should target not only sustained hyperglycemia, but also glucose excursion.

In nuclear and mitochondrial DNA, 8-OH-dG, an oxidized nucleoside of DNA, is the most frequently detected and studied DNA lesion [21]. While studies have shown

severe oxidative DNA damage in diabetes, especially in patients with diabetic complications, the effect of glucose fluctuation on the activation of DNA oxidation has not been reported to our knowledge [16, 17]. More importantly, in this study, no significant relationship was found between HbA1c and plasma 8-OH-dG level, even in patients with T2DM ($P > 0.05$). Further analysis by multiple linear regression confirmed that postprandial hyperglycemic spikes estimated from MPPGE exhibit a stronger triggering effect on oxidative DNA damage than chronic sustained hyperglycemia estimated from HbA1c in both subjects with IGR and T2DM. While these results do not provide direct evidence that postprandial glucose spikes induce oxidative DNA damage, the triggering effect of postprandial glucose spikes cannot be ignored and deserves further investigation. In fact, many studies have shown that 8-OH-dG levels are a risk factor for atherosclerosis in diabetes. Furthermore, several authors have speculated that augmented oxidative DNA damage in diabetes contributes to the pathogenesis of diabetic complications [14, 22, 23].

Carbonyl group formation is considered an early and stable marker for protein oxidation. There is evidence that protein oxidation induced by glucose may cause inactivation of antioxidant defense enzymes and damage in the structure and function of plasma protein [24, 25]. In the present study, we found higher plasma levels of protein carbonyl in patients with newly diagnosed T2DM and in those with IGR compared to normal controls, an observation consistent with other reports [26, 27]. Another report suggests that exposure to postprandial hyperglycemia induces the overproduction of oxygen-free radicals and, consequently, increases the production of protein oxidation [28]. Our multivariate analysis showed that the 2hPG measurement and, to a lesser extent, IAUC, were independent determinants of plasma protein carbonyl levels in study subjects with abnormal glucose metabolism. To our knowledge, this is the first report on the effect of glucose excursion on protein oxidation in diabetes. Although the primary pathophysiological mechanism by which postprandial glucose excursions activate the process of protein oxidation remains unknown, our results suggest that postprandial hyperglycemia, as well as glucose excursions by meals, might contribute to the development of diabetic complication through carbonyl stress, even during prediabetic stages.

In this study, we measured oxidative stress markers in plasma samples collected at a fixed time point. Admittedly, these values may underestimate the fluctuations in oxidative stress levels that might be found in a 24-h urine sample. However, we were able to demonstrate a strong relationship between glucose excursions and the activation of oxidative stress both in subjects with newly diagnosed T2DM and subjects with IGR. Therefore, reducing glucose fluctuations in patients with T2DM, as well as in those with IGR, is important for minimizing vascular damage brought about through oxidative stress.

Materials and methods

Subjects

All subjects were informed about the nature and the risks of the study procedures before their written informed consent was obtained. Study participants were classified based on FPG and 2hPG levels following oral glucose tolerance test (OGTT). A total of 30 individuals with NGR were enrolled, having FPG < 6.1 mmol/l and 2hPG < 7.8 mmol/l. Twenty-seven subjects with IGR were enrolled, including 18 subjects with impaired glucose tolerance (IGT), having FPG < 6.1 mmol/l and $7.8 \leq$ 2hPG < 11.1 mmol/l, and 9 subjects with impaired fasting glucose (IFG) + IGT, having $6.1 \leq$ FPG < 7.0 mmol/l and $7.8 \leq$ 2hPG < 11.1 mmol/l. Twenty-seven additional patients with newly diagnosed type 2 diabetes (T2DM) were ascertained through the endocrine clinic of Sir Run Run Shaw Hospital (Hangzhou, China). Diagnosis of diabetes was made according to the World Health Organization criteria of 1999. All diabetic patients had not been treated with oral antidiabetic agents, and HbA1c levels of these patients were <9%. The clinical characteristics of all study subjects are shown in Table 1. Exclusion criteria for this study included impaired renal or liver function, history of angina pectoris, history of cerebral or myocardial infarction, treatment with statins, steroids, or nonsteroidal anti-inflammatory drugs, or treatment with antioxidative stress drugs such as Vitamin C and Vitamin E.

CGMS monitoring

Subcutaneous interstitial glucose levels were monitored by CGMS (Medtronic MiniMed, Northridge, CA) over a period of three consecutive days. The sensor was inserted on day 0 and removed on day 3. During the 72-h CGMS period, all the subjects received a large calorie meal, totaling 30 kJ/kg of body weight and consisting of 60 energy-percent (En%) carbohydrate, 25 En% fat, and 15 En% protein at the set meal points (breakfast about 20%, lunch 40%, dinner 40% of total daily energy). Daily energy expenditures were approximated, and detailed written records of any particular event (food intake and exercise) were coded into the monitor. At least four daily self-monitoring capillary blood glucose measurements were obtained with a glucometer (OneTouch® Ultra®, Johnson & Johnson, USA) were entered into the CGMS monitor (CGMS, Medtronic MiniMed, Northridge, CA) for

calibration. Following the CGMS period, all the subjects returned to the hospital, and CGMS data were downloaded via the Com-Station using the MiniMed Solutions Software version 3.0 (MiniMed, Sylmar, CA).

Assessment of glycemic condition

The mean blood glucose value (MBG) was calculated as the arithmetic mean of 288 glucose values within a 24-h period. Several parameters were used to assess intra- and inter-day glycemic variabilities. Calculation of the MAGE was obtained by measuring the arithmetic mean of the differences between consecutive peaks and nadirs having an amplitude >1 SD of MBG for a 24-h period. Measurement in the peak to nadir or nadir to peak was determined by the first qualifying excursion [15]. A higher MAGE might suggest higher glycemic instability intra-day. Mean postprandial glucose excursion (MPPGE) was the arithmetic mean of the three differences between the postprandial peak glucose values and the corresponding pre-prandial glucose values over a 24-h period. The mean postprandial incremental area under the curve (IAUC) above three pre-prandial glucose values were calculated over a 3-h period following the beginning of each meal, and then, three incremental areas were summed and averaged [29]. MPPGE and IAUC were used for assessment of postprandial glucose excursions. All parameters were averaged on study days 1 and 2 (i.e, from continuous monitoring for 48 h) to calculate the mean values. The mean of daily differences (MODD) was calculated as the mean of the absolute values of the difference between glucose values taken on two consecutive days at the same time. A higher MODD might suggest a higher glycemic instability inter-day [30].

Sample collection and laboratory measurements

For the OGTT, fasting and 2 h blood samples were taken from the cubital vein. Serum triglycerides (TG), total cholesterol, plasma glucose concentrations, and serum and urinary creatinine were analyzed using enzymatic techniques, and HbA1c was measured with fast performance liquid chromatography (Bio-Rad). High-density lipoprotein (HDL) cholesterol concentration was quantified using polyethylene glycol-modified enzymes and low-density lipoprotein (LDL) cholesterol concentration was calculated using the formula of Friedewald.

Plasma and urine samples were also collected and assessed for signs of oxidative stress. Postprandial plasma samples were collected from all the subjects on day 3 of the CGMS period. Three hours after a breakfast of 100 g bread, blood samples were collected in EDTA-2Na tubes. Plasma were separated and stored at −70°C until analysis. Due to scheduling conflicts, only 10 subjects were able to collect 24 h urine samples. Plasma and urinary 8-iso-PGF2α and 8-OH-dG concentrations were determined using EIA kits (Cayman Chemical, USA). The intra- and inter-assay coefficients of variation were within 10%. Plasma protein carbonyl content was determined using a dinitrophenyhydrazine (DNPH) colorimetric assay kit (Cayman Chemical, USA). The intra-assay coefficient of variation was 5.8% and the inter-assay coefficient of variation was 10%.

Statistical analyses

Data were analyzed with SPSS 13.0. Variables with normal distribution are presented as mean ± SD, and skewed variables (plasma 8-iso-PGF2α and 8-OH-dG) were presented as median and quartile range M (QR) and ln-transformed to reach normal distribution. Differences among subjects in different glucose regulation categories were tested by one-way ANOVA, Student's unpaired *t* test, or Chi-square test. Linear regression models in multivariate analyses were used to adjust for the potential confounders such as BMI, waist circumference, and HbA1c. Correlations between continuous variables with normal distribution were tested with Pearson's correlation analysis. Multiple stepwise linear regression models were used to explore the effects of different variables on the activation of oxidative stress. The *F* value for the inclusion and exclusion of variables was set at 4.0 at each step. Statistical significance was defined as $P < 0.05$.

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