Oscillating Glucose Is More Deleterious to Endothelial Function and Oxidative Stress Than Mean Glucose in Normal and Type 2 Diabetic Patients

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OBJECTIVE—To explore the possibility that oscillating glucose may outweigh A1C levels in determining the risk for cardiovascular diabetes complications.

RESEARCH DESIGN AND METHODS—A euinsulinemic hyperglycemic clamp at 5, 10, and 15 mmol/l glucose was given in increasing steps as a single "spike" or oscillating between basal and high levels over 24 h in normal subjects and type 2 diabetic patients. Flow-mediated dilatation, a marker of endothelial function, and plasma 3-nitrotyrosine and 24-h urinary excretion rates of free 8-iso PGF2α, two markers of oxidative stress, were measured over 48 h postclamp.

RESULTS—Glucose at two different levels (10 and 15 mmol/l) resulted in a concentration-dependent fasting blood glucose–independent induction of both endothelial dysfunction and oxidative stress in both normal and type 2 diabetic patients. Oscillating glucose between 5 and 15 mmol/l every 6 h for 24 h resulted in further significant increases in endothelial dysfunction and oxidative stress compared with either continuous 10 or 15 mmol/l glucose.

CONCLUSIONS—These data suggest that oscillating glucose can have more deleterious effects than constant high glucose on endothelial function and oxidative stress, two key players in favoring cardiovascular complications in diabetes. Concomitant vitamin C infusion can reverse this impairment. ***Diabetes*** **57: 1349–1354, 2008**

A strong relationship between the mean levels of glycemia, measured as A1C, and diabetes complications (1–2), including cardiovascular complications (3), has been demonstrated. However, what is still unclear is whether glycemic instability may confer additional risk to the development of complications over that predicted by the mean glucose value alone. It is therefore unknown whether two individuals with the same mean blood glucose but extremes of glucose variability might have the same or different level of risk for complications.

In a 1995 report of the Diabetes Control and Complications Trial, the risk of retinopathy progression associated with a given level of mean A1C differed significantly between intensively and conventionally treated patients (4). It was suggested that this may be a consequence of larger glycemic excursions in the conventional group (4). However, with regard to cardiovascular complications, it has been reported that fasting plasma glucose instability is a predictor of cardiovascular-related mortality in elderly patients with type 2 diabetes, and it has been suggested that glucose stability might be a goal in the management of these patients (5). A related issue is the evidence that postprandial glycemia is a stronger risk marker for macrovascular complications than fasting glucose (6).

Endothelial dysfunction is predictive of a future cardiovascular event (7), and evidence indicates that hyperglycemia induces endothelial dysfunction through an oxidative stress (8), which is a key player in the development of diabetes complications (9). The aim of this study is to verify the effect of glucose variability on endothelial function and the possible involvement of oxidative stress in the phenomenon.

RESEARCH DESIGN AND METHODS

Twenty-seven type 2 diabetic patients and 22 healthy subjects were recruited (Table 1). The diabetic patients had newly diagnosed (within 6 months) type 2 diabetes, were on diet alone, and had no evidence of any cardiovascular complications. The protocol of the study was approved by the ethics committee of our institution. All subjects gave informed consent before being tested. The subjects of this study underwent periods of hyperglycemia and periods of normoglycemia. Below, the techniques used to attain these conditions are described.

To maintain euinsulinemia, endogenous insulin secretion was inhibited during all the experiments using somatostatin (Sandostatin; Novartis Pharma, Basel, Switzerland) (10). Somatostatin was infused in two phases: *1*) as a bolus dose of 25 μg over 1 min given 5 min before the start of the experiment and *2*) as a continuous maintenance dose of 1.0 μg/min (10). The hyperglycemic-euinsulinemic clamp metholody was a modification of the method used by Del Prato et al. (10).

Normalization of glycemia. Insulin and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/l were started (11). Blood glucose levels were determined every 5 min with adjustment of the intravenous insulin infusion until steady-state glucose levels were between 4 and 6 mmol/l. At the steady state, venous glucose samples were drawn every 30 min.

Control study with somatostastin. Five normal subjects and five diabetic patients underwent a control study with 3 h somatostatin infusion alone, and endothelial function and nitrotyrosine plasma levels were evaluated. No change in these parameters was found.

Protocols. Two different clamp protocols were planned, and the diabetic patients and the normal subjects participating in each protocol were matched for age, sex, and BMI and for fasting glycemia and A1C in the case of diabetes.

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FMD, flow-mediated dilatation; PGF2α, prostaglandin F2α.

TABLE 1
Baseline characteristics of normal and diabetic subjects

	Control subjects	Diabetic subjects
n	22	27
Sex (male/female)	12/10	14/13
Age (years)	50.5 ± 2.5	51.3 ± 2.6
BMI (kg/m^2)	28.5 ± 3.1	29.5 ± 3.3
Fasting glucose (mmol/l)	4.5 ± 0.3	7.8 ± 2.2*
A1C (%)	4.8 ± 0.2	7.7 ± 0.3*
Resting systolic blood pressure (mmHg)	117.3 ± 5.5	123.4 ± 6.4
Resting diastolic blood pressure (mmHg)	77.5 ± 2.2	80.2 ± 3.6
Total cholesterol (mmol/l)	4.5 ± 0.6	5.1 ± 0.8
Triglycerides (mmol/l)	0.9 ± 0.2	1.2 ± 0.4
HDL cholesterol (mmol/l)	1.4 ± 0.2	1.2 ± 0.3
LDL cholesterol (mmol/l)	2.5 ± 0.3	2.6 ± 0.4
FMD (%)	11.7 ± 0.7	5.9 ± 0.6*
Nitrotyrosine (μmol/l)	0.24 ± 0.5	0.52 ± 0.3*

Data are means ± SEM unless otherwise indicated. *$P < 0.001$ vs. control subjects.

In both of the protocols, 24-h urinary excretion rate of free 8-iso prostaglandin F2α (PGF2α) was also measured.

Oscillating high and normal glucose. This protocol was designed to explore the direct effect of glucose variability compared with stable hyperglycemia on endothelial function and oxidative stress production. In 12 normal subjects and in 15 diabetic patients, in randomized order, for 24 h, glycemia was increased at 15 mmol/l every 6 h and normalized for the next 6 h, maintained at 15 mmol/l (peak value), and maintained at 10 mmol/l (mean glycemia value per 24 h of experiment one). Flow-mediated dilatation (FMD) and nitrotyrosine were measured every 6 h during the experiments and 24 h after the experiment end.

Oscillating glucose plus vitamin C. This protocol was designed to explore the effect of oxidative stress on the endothelial dysfunction induced by oscillating glucose. In 10 normal subjects and in 12 diabetic patients, in randomized order, for 24 h, glycemia was increased by 15 mmol/l every 6 h and normalized for the next 6 h with or without vitamin C infusion (3 mg/min) (11). FMD and nitrotyrosine were measured every 6 h during the experiments and 24 h after the experiment end.

Biochemical measurements. Plasma glucose, cholesterol, triglycerides, HDL cholesterol, and A1C were measured by routine laboratory methods. LDL cholesterol was calculated after lipoprotein separation (12). Nitrotyrosine was measured by enzyme-linked immunosorbent assay (13). Free 8-iso PGF2α plasma levels were measured by a commercially available kit (Cayman Chemical, Ann Arbor, MI).

Endothelial function. Endothelial function was evaluated using flow-mediated dilatation of the brachial artery. The validity of this method has been confirmed in previous studies (14–15). At the end of each test, the subjects lay quietly for 15 min. Then, sublingual nitroglycerine (0.3 mg) was administered and, 3 min later, the last measurements were performed. Response to nitroglycerine was used as a measure of endothelium-independent vasodilatation. In this study we were able to confirm, as previously reported, that interobserver variability for repeated measurements of resting arterial diameter was 0.04 ± 0.02 mm, while the intra-observer variability for repeated measurements of resting arterial diameter was 0.02 ± 0.02 mm (8).

Statistical analysis. The number of subjects to include in each protocol was selected according to the guidelines in previous available literature (16–19). The Kolmogorov-Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data among the groups were performed using unpaired Student's *t* test. The changes in variables during the tests were assessed by two-way ANOVA with repeated measures. If differences reached statistical significance, post hoc analyses with two-tailed paired *t* test were used to assess differences at individual time periods in the study, using Bonferroni correction for multiple comparisons. Statistical significance was defined as $P < 0.05$. Data are reported as means ± SEM.

RESULTS

The baseline differences between diabetic and normal subjects are reported in Table 1. For the oscillating high and normal glucose compared with high constant glucose protocol, clamping glycemia at 10 and 15 mmol/l produced an impairment of endothelial function ($P < 0.01$ vs. basal at any time) and an increase in nitrotyrosine generation ($P < 0.01$ vs. basal at any time) in both normal and diabetic patients (Figs. 1 and 2).

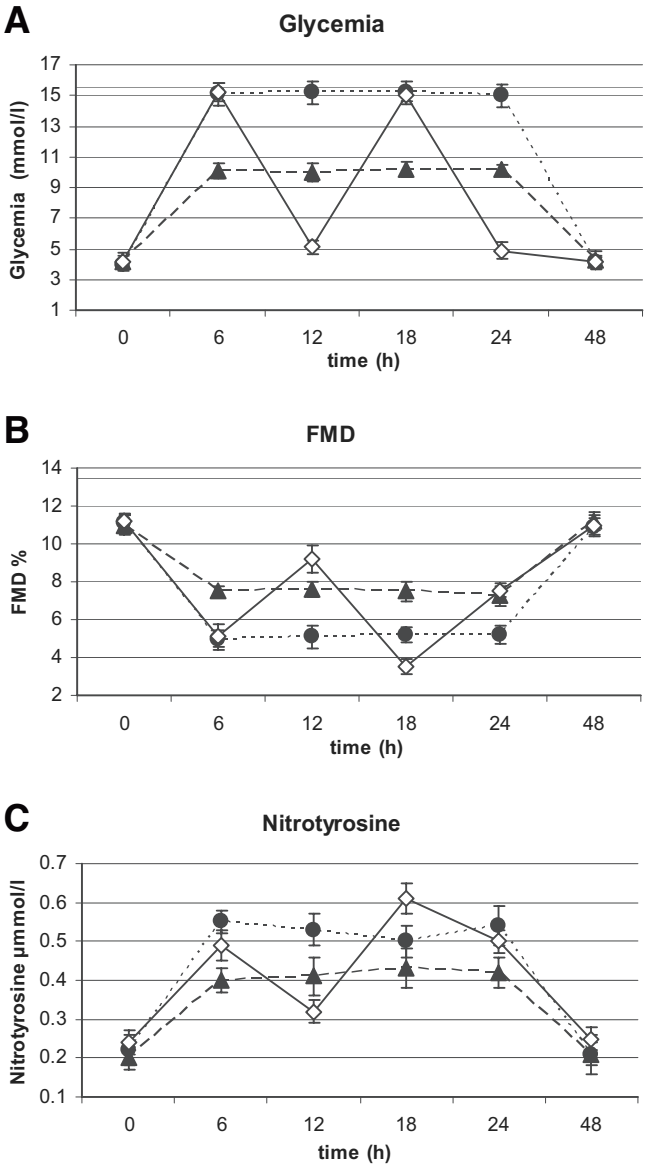

FIG. 1. ***A*: Hyperglycemic clamps in normal subjects. For 24 h, glycemia *1*) was increased at 15 mmol/l every 6 h and normalized for the next 6 h (◇), *2*) maintained at 15 mmol/l (●) (peak value), and *3*) maintained at 10 mmol/l (▲) (mean glycemia value/24 h of experiment 1). *B* and *C*: FMD and nitrotyrosine measured during the experiments and 24 h after the end. Bars indicate SEM.**

Normal subjects. Endothelial dysfunction ($P < 0.01$ at any time) and nitrotyrosine levels ($P < 0.01$ at any time, except at 18 h, when P = NS) were significantly increased in response to 15 compared with 10 mmol/l glucose. Oscillation of glycemia from basal levels to 15 mmol/l produced significant endothelial dysfunction (at 6 h, $P < 0.01$ vs. basal; at 18 h, $P < 0.001$ vs. basal) and increase in nitrotyrosine levels (at 6 h, $P < 0.01$ vs. basal; at 18 h, $P < 0.001$ vs. basal). In oscillating glucose compared with constant glucose at 15 mmol/l, after the first 6 h of hyperglycemia, levels of endothelial dysfunction and nitro-

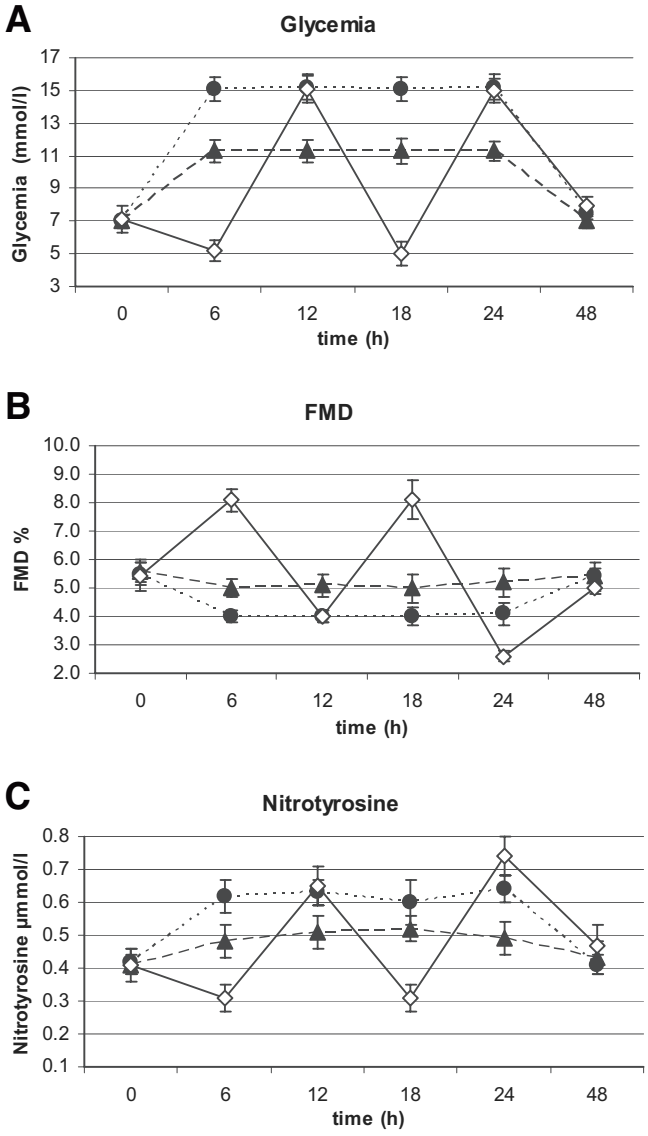

FIG. 2. Hyperglycemic clamps in diabetic patients. For 24 h, glycemia *1*) was increased at 15 mmol/l every 6 h (◇), *2*) maintained at 15 mmol/l (●) (peak value), and *3*) maintained at 10 mmol/l (▲) (mean glycemia value/24 h of experiment 1). *B* and *C*: FMD and nitrotyrosine measured during the experiments and 24 h after the end. Bars indicate SEM.

tyrosine were superimposable (P = NS). At 18 h, however, after the second period of hyperglycemia, both endothelial dysfunction ($P < 0.01$) and nitrotyrosine levels ($P < 0.01$) were higher in the oscillating glucose. The effect of oscillating glucose compared with 10 mmol/l constant glucose was even more pronounced than the 15 mmol/l results for both endothelial dysfunction and nitrotyrosine (6 and 18 h, $P < 0.01$). During the oscillating period, even when glycemia was normal, neither endothelial function nor nitrotyrosine returned to the basal levels. The data are reported in Fig. 1.

Diabetic patients. Endothelial dysfunction ($P < 0.05$ at any time) and nitrotyrosine levels ($P < 0.01$ at any time, except at 18h P = NS) were significantly increased in response to 15 as compared to 10 mmol/l constant glucose in diabetic patients. At 12 h, in oscillating the situation, after the first period of hyperglycemia, both endothelial dysfunction and nitrotyrosine were superimposable to the levels observed at the same time point in response to 15 mmol/l constant high glucose and significantly different from those observed at 10 mmol/l ($P < 0.05$ and $P < 0.01$, respectively) at 12 h. At 24 h, however, endothelial dysfunction was significantly increased compared with that in both 10 and 15 mmol/l constant glucose groups ($P < 0.01$ vs both), while nitrotyrosine was significantly increased compared with that in the 10 mmol/l ($P < 0.001$) but not the 15 mmol/l (P = NS) group. In diabetic subjects, compared with both the 10 and 15 mmol/l constant glucose groups, normalizing glucose levels in the oscillating group resulted in improved endothelial dysfunction and nitrotyrosine levels. The data are reported in Fig. 2.

The 24-h urinary excretion rates of free 8-iso PGF2α were significantly higher in the oscillating glucose groups compared with either the constant high 15 mmol/l or 10 mmol/l groups in both normal (342 ± 52 vs. 271 ± 54 pg/mg creatinine [$P < 0.05$] and vs. 230 ± 35 pg/mg creatinine [$P < 0.01$]) and diabetic (536 ± 51 vs. 476 ± 48 pg/mg creatinine [$P < 0.05$] and vs. 432 ± 47 pg/mg creatinine [$P < 0.01$]) patients.

Oscillating glucose plus vitamin C. In the normal subjects at 6 h and 18 h, both endothelial dysfunction ($P < 0.01$ and $P < 0.001$, respectively) and nitrotyrosine levels ($P < 0.01$ and $P < 0.001$, respectively) were significantly different in response to oscillating glucose compared with those at basal time points. The values of both endothelial function and nitrotyorisine were at 18 h significantly different from those at 6 h ($P < 0.01$) in response to oscillating glucose. The infusion of vitamin C counterbalanced the effect of oscillating glucose (Fig. 3).

In the diabetic patients at 12 h and 24 h, both endothelial dysfunction ($P < 0.01$ and $P < 0.001$, respectively) and nitrotyrosine levels ($P < 0.01$ and $P < 0.001$, respectively) were significantly different compared with those at basal time points in response to oscillating glucose. The values of both endothelial function and nitrotyorisine were at 24 h significantly different from those at 12 h ($P < 0.01$) in response to oscillating glucose. The infusion of vitamin C counterbalanced the effect of oscillating glucose (Fig. 4).

However, in normal subjects, vitamin C not only counterbalanced the effect of oscillating glucose but almost normalized both endothelial function and nitrotyrosine levels (Fig. 3). In diabetic patients, vitamin C, during high glucose periods, maintained endothelial function and nitrotyrosine at basal levels; however, during the periods of glucose normalization, vitamin C further improved, compared with those at baseline, both endothelial dysfunction and nitrotyrosine levels (Fig. 4).

The 24-h urinary excretion rates of free 8-iso PGF2α were significantly higher in the oscillating glucose group without vitamin C infusion in both normal (351 ± 88 vs. 160 ± 55 pg/mg creatinine, $P < 0.001$) and diabetic (550 ± 78 vs. 320 ± 78 pg/mg creatinine, $P < 0.01$) patients. Endothelium-independent vasodilation did not change during any of the tests (data not shown).

DISCUSSION

In this study, we have been able to show that oscillating glucose, over a period of 24 h, is more damaging to endothelial function than stable constant high glucose. This is true not only when a subject is exposed to the same total amount of glucose for 24 h (i.e., 10 mmol/l clamp) but even when the total amount is higher (i.e., 15 mmol/l clamp). Finally, data suggest that oxidative stress plays a key role in all of these phenomena.

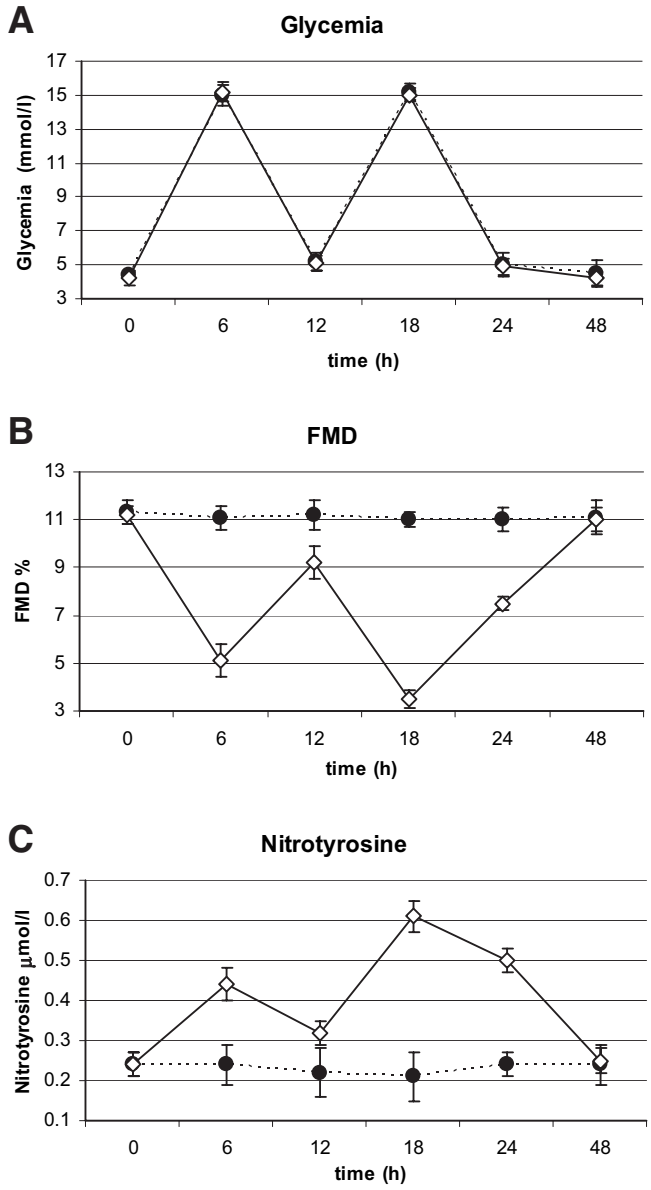

FIG. 3. Hyperglycemic clamps in normal subjects. *A*: For 24 h, glycemia was increased at 15 mmol/l every 6 h and normalized for the next without (◇) or with (●) mg/m vitamin C infusion. *B* and *C*: FMD and nitrotyrosine measured during the experiments and 24 h after the end. Bars indicate SEM.

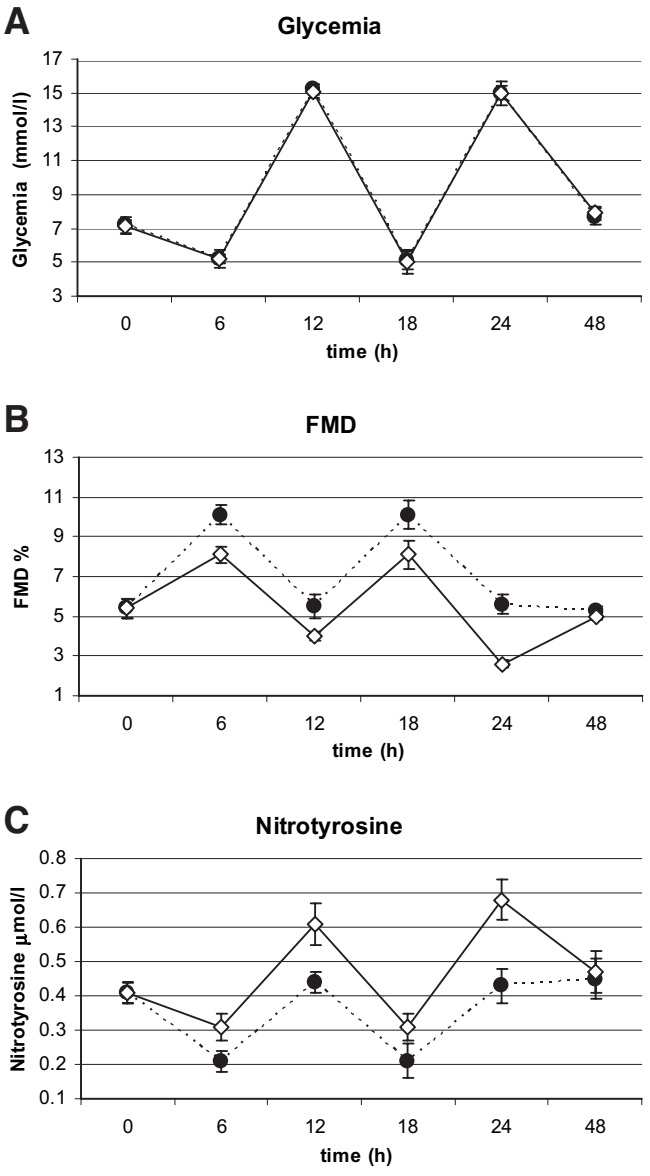

FIG. 4. Hyperglycemic clamps in diabetic patients. *A*: For 24 h, glycemia was increased at 15 mmol/l every 6 h and normalized for the next without (◇) or with (●) mg/m vitamin C infusion. *B* and *C*: FMD and nitrotyrosine measured during the experiments and 24 h after the end. Bars indicate SEM.

Many reports have already shown that an acute increase of glycemia induces endothelial dysfunction in both normal and diabetic subjects (8,16–19). However, in these experiments only the effect of a single increase, for a few hours, of glycemia has been evaluated. For the first time, we can show that fluctuations of glycemia are always accompanied by an impact on endothelial function. This effect seems to be related to the level of glycemia reached. This is not surprising, in that a strong direct correlation has already been demonstrated between endothelial function and the level of glycemia reached during an oral glucose tolerance test in normal subjects and in individuals with impaired glucose tolerance and with overt diabetes (19) or when hyperglycemia is differently managed in the postprandial state (20–21).

Again for the first time, we are able to show in humans that the effect of oscillating glucose is worse than that of constant high glucose. This concept has been widely demonstrated in both cells and animals (22–25). Interestingly, in our study the effect is independent of the total amount of glucose to which subjects are exposed. Similar results have already been obtained in vitro in human renal cortical fibroblasts (26).

Even if oxidative stress generation appears to be the key player of all the phenomena observed in the study, the precise mechanism through which oscillating glucose may be worse than constant high glucose remains incompletely defined. Although further studies are certainly warranted, these would be quite difficult to accomplish in humans. Studies in cells and in animals, however, have shown that in oscillating glucose pathways involving protein kinase C (22), NADPH (22), inducible nitric oxide synthase (27), or inflammatory markers are more activated in response to oscillating glucose compared with constant high glucose (23–25). However, it has also been shown that the activa-

tion of many of these pathways is secondary to the generation of free radicals at the level of the mitochondria during high glucose overload (23,28). As preliminarily reported, another possible explanation is that in oscillating glucose conditions the cells are not able in sufficiently increase their own intracellular antioxidant defenses (29), a condition which has been suggested to favor the development of diabetes complications (30–31). In this regard, a recent study showed that during acute hyperglycemia, in normal subjects, several genes involved in free radical detoxification were downregulated (32).

Even though there was an absence of significance in the nitrotyrosine levels at 24 h between 15 mmol/l constant glucose and oscillating glucose in diabetic patients, overall in this study, nitrotyrosine plasma levels were found to change according to the variations in glycemia in both normal and diabetic subjects, as has previously been published (8,11,33). Other factors, such as the activation of inflammation, could be involved; however, we are able to confirm that glucose oscillation is accompanied by an increased excretion of free 8-iso PGF2α in the 24 h of each study (34). Even in the case of this marker, the oscillation of glucose is more harmful than constant high glucose. Finally, almost all the alterations induced by high glucose, either stable or oscillating, are reversed by vitamin C.

An apparent limitation of this study is that a significant difference between oscillating glucose and the constant infusion of glucose can be observed only in the second pulse of oscillating glucose. In our opinion, this is exactly what we can expect from the design of the study. In fact, the first pulse of high glucose can simply show the effect of an acute increase of glycemia, as reported in many other studies. The effect of oscillation in glucose can be observed, by definition, only after the first pulse. More frequent pulses of glucose would better confirm the hypothesis; however, this kind of design could be very difficult to carry out.

The involvement of oxidative stress merits attention. In diabetes, free radical overproduction is not only involved in generating endothelial dysfunction (35) and linked to an increased risk of cardiovascular disease (36) but, specifically in this disease, it is strictly related to the development of complications (9). More specifically, it is certainly intriguing that nitrotyrosine (37) and free 8-iso PGF2α (38) are both independent risk factors for cardiovascular disease.

Other findings of this study may deserve attention. During the oscillating experiments, in normal subjects endothelial function and nitrotyrosine never returned to basal levels, while this happened in diabetic patients. Furthermore, vitamin C was able to normalize endothelial function and oxidative stress in normal subjects but not in diabetic subjects during the period of hyperglycemia, whereas when glycemia was normalized in diabetic patients, the simultaneous administration of vitamin C almost normalized both endothelial function and oxidative stress. A possible hypothetical explanation is that two pathways are simultaneously working: one due to the actual level of glycemia and another one to the long-lasting damage induced in the endothelial cells by chronic hyperglycemia, possibly through nonenzymatic glycation of mitochondria (39). Although speculative, this explanation is consistent with a previous finding showing that only the simultaneous control of hyperglycemia together with vitamin C infusion can normalize endothelial function in diabetes (11).

Finally, a possible role for insulin, more than for the reduction of hyperglycemia, in determining the improvement of endothelial dysfunction might be supposed. However, even though Ellger et al. have recently shown in an animal model that it is the reduction of glucose toxicity more than the action of insulin that improves endothelial function (40), the role of insulin as a protector against activation of oxidative stress cannot be completely excluded. Additional studies, such as the assessment of the relationship between markers of oxidative stress and insulin concentrations during an euglycemic-hyperinsulinemic clamp, are needed.

Our results support the hypothesis that the oscillation of plasma glucose may impact on endothelial function and oxidative-stress generation more than stable high glucose, two well-recognized risk factors in diabetes for cardiovascular complications (35). Even though, in light of these results, specific trials are needed for a final answer, we trust that the results of this study may contribute not only in giving a possible pathophysiological explanation of the phenomenon but also in raising the alert for a need to avoid glucose oscillation in clinical practice to prevent cardiovascular diabetes complications.

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