Inflammatory Cytokine Concentrations Are Acutely Increased by Hyperglycemia in Humans
Role of Oxidative Stress

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Background—Circulating levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are elevated in diabetic patients. We assessed the role of glucose in the regulation of circulating levels of IL-6, TNF-α, and interleukin-18 (IL-18) in subjects with normal or impaired glucose tolerance (IGT), as well as the effect of the antioxidant glutathione.

Methods and Results—Plasma glucose levels were acutely raised in 20 control and 15 IGT subjects and maintained at 15 mmol/L for 5 hours while endogenous insulin secretion was blocked with octreotide. In control subjects, plasma IL-6, TNF-α, and IL-18 levels rose (P<0.01) within 2 hours of the clamp and returned to basal values at 3 hours. In another study, the same subjects received 3 consecutive pulses of intravenous glucose (0.33 g/kg) separated by a 2-hour interval. Plasma cytokine levels obtained at 3, 4, and 5 hours were higher (P<0.05) than the corresponding values obtained during the clamp. The IGT subjects had fasting plasma IL-6 and TNF-α levels higher (P<0.05) than those of control subjects. The increase in plasma cytokine levels during the clamping lasted longer (4 hours versus 2 hours, P<0.01) in the IGT subjects than in the control subjects, and the cytokine peaks of IGT subjects after the first glucose pulse were higher (P<0.05) than those of control subjects. On another occasion, 10 control and 8 IGT subjects received the same glucose pulses as above during an infusion of glutathione; plasma cytokine levels did not show any significant change from baseline after the 3 glucose pulses.

Conclusions—Hyperglycemia acutely increases circulating cytokine concentrations by an oxidative mechanism, and this effect is more pronounced in subjects with IGT. This suggests a causal role for hyperglycemia in the immune activation of diabetes. (Circulation. 2002;106:2067-2072.)

Key Words: hyperglycemia • tumor necrosis factor • interleukins • glutathione

Type 2 diabetes mellitus is associated with an increased risk of premature atherosclerosis: Coronary artery disease, cerebrovascular disease, or peripheral vascular disease is the cause of death in 75% to 80% of adult diabetic subjects.1 Although the conviction that hyperglycemia plays a role in the pathogenesis of cardiovascular complications in diabetic patients has waxed and waned in recent years, both prospective studies on the relationship between plasma glucose and cardiovascular events and clinical trials of intensive glucose control have found a link between high glucose levels and cardiovascular diseases, without any apparent threshold.2

It has been postulated that type 2 diabetes mellitus may represent a disease of the innate immune system responsible for an ongoing cytokine-mediated acute phase response.3 Consistent with this hypothesis, a prospective study found that two circulating markers of systemic inflammation, C-reactive protein and interleukin-6 (IL-6), were determinant of risk for development of type 2 diabetes mellitus in apparently healthy middle-aged women.4 Moreover, several studies have demonstrated elevated levels of IL-6 and tumor necrosis factor-α (TNF-α) among individuals both with features of the insulin resistance syndrome and with clinically overt type 2 diabetes mellitus.5–8

The aim of the present study was to test whether circulating levels of cytokines are regulated by glucose levels in humans. The study protocol was designed to measure serum TNF-α, IL-6 and interleukin-18 (IL-18) concentrations during acute hyperglycemia in subjects with normal or impaired glucose tolerance (IGT); a role for IL-18 in plaque destabilization has recently been suggested,9 and stress hyperglycemia is an important risk factor for post–myocardial infarction mortality in subjects with or without diabetes.10 Because oscillatory hyperglycemia may be more toxic for endothelial cells than continuous hyperglycemia,11,12 we also assessed the cytokine...
behavior in response to consecutive glucose pulses. Finally, we tested the effect of the antioxidant glutathione on cytokine responses to acute hyperglycemia; there is evidence that the release of TNF-α induced by high glucose in vitro may be mediated by reactive oxygen species 13 and that oxidative stress might be implicated in promoting a low-grade systemic inflammation in patients with type 2 diabetes mellitus.6

Methods

Study Population

The control subjects were recruited from the medical and paramedical staff of the Department of Geriatrics and Metabolic Diseases at the Second University of Naples. Subjects with IGT were selected from the type 2 diabetic patients’ first-degree relatives who presented a 2-hour plasma glucose value between 7.7 and 11 mmol/L after a standard 75-g oral glucose tolerance test. The clinical and metabolic characteristics of all subjects are shown in the Table. The control subjects had normal glucose tolerance (2-hour plasma glucose value below 7.7 mmol/L) and no evidence of hypertension, hyperlipidemia, or any systemic conditions; the IGT subjects had no evidence of any cardiovascular complications. All subjects were on weight-maintaining diets with 250 g of carbohydrate per day, had no recent change in body weight or intercurrent illness, and were taking no medications. Particular care was taken to exclude subjects with infections or inflammatory diseases, as confirmed with measurement of C-reactive protein level of <5 mg/L. None of the subjects were engaged in physical activity for >3 hours per week or smoked. The ethics committee of our institution approved the protocol of the study. All subjects gave informed consent before being tested.

Study Protocol

After a 12-hour overnight fast, subjects were placed in a supine comfortable position with a room temperature between 20° and 24°C. Intravenous lines were inserted into a large antecubital vein of one arm for infusions and into a dorsal vein of the contralateral arm for blood sampling. Patency was preserved by a slow saline infusion (0.9% NaCl). The study began after the subjects had rested for 30 minutes. The subjects underwent the tests in random order and separated by at least a 3-day interval.

Study 1

All subjects participated in the glucose clamp study. After withdrawal of baseline blood samples, plasma glucose concentrations were acutely raised with a bolus injection of 0.33 g/kg glucose followed by a varying 30% glucose infusion to achieve steady-state plasma glucose concentrations of about 15 mmol/L for 240 minutes. This test was performed with the aid of an artificial pancreas (Biostator, Life Science), which allowed exogenous glucose to be infused in order to match the prefixed plasma glucose value on the basis of continuous blood glucose monitoring. Octreotide (25 μg IV bolus followed by a 0.5 μg/min infusion, Longastatina; Italfarmaco) was infused to block the release of endogenous insulin. The octreotide infusion was started 5 minutes before the priming glucose pulse and was interrupted at the end of the clamp. To prevent hypokalemia, 0.26 mmol/L KCl was added to the glucose infusion.

To exclude any direct effect of octreotide on cytokine levels, 5 control and 5 IGT subjects received an additional infusion of octreotide alone at the dose administered earlier, in the absence of the hyperglycemic clamp.

Study 2

All subjects participated in the glucose pulse study. In this study, three consecutive bolus of intravenous glucose (0.33 g/kg) were injected in a large antecubital vein separated by a 2-hour interval. After the first glucose pulse, plasma glucose levels had returned to baseline before the administration of the subsequent glucose pulse. Even in this study, and as described earlier, an octreotide infusion was started 5 minutes before the first glucose pulse and was interrupted 60 minutes after the third glucose pulse, ie, 5 hours later.

Study 3

Ten (5 male, 5 female) control and 8 (4 male, 4 female) IGT subjects participated in the gluthane study. This study consisted of the consecutive glucose pulses as described in study 2 plus glutathione infusion (600 mg as an intravenous bolus followed by a 5-mg/min infusion). Both octreotide and glutathione infusions were started 5 minutes before the first glucose pulse and lasted 5 hours.

Analyses

Samples for analysis of plasma glucose were collected in tubes containing a trace of sodium fluoride, and samples for analysis of insulin were collected in tubes containing a mixture (0.1 mL/mL blood) of EDTA-aprotinin (TrasyloL solution) (500 U/mL Trasylol [Bayer], 1.2 g/L disodium EDTA). Plasma glucose was determined according to the glucose oxidase method with an autoanalyzer (Beckman Instruments). Labile and stable forms of glycéto hemoglobin A1 (HbA1) were determined in duplicate as previously described.14 Plasma insulin levels were determined with radioimmunoassay.15 Serum samples for cytokines were stored at ~80°C until assay. Serum concentrations of TNF-α, IL-6, and IL-18 were determined in duplicate with commercially available kits (R&D Systems). Dilution curves of serum samples were parallel those of standard. Intra-assay and interassay coefficients of variation were 3.8% and 5.8%, respectively, for TNF-α; 3.9% and 5.9%, respectively, for IL-6; and 3.5% and 6.1%, respectively, for IL-18.

Statistical Analysis

Results are given as mean±SD. One-way ANOVA was used to compare baseline data, followed by Scheffé’s test for pairwise comparisons. Multiple comparison tests were made with ANOVA, followed by post hoc analysis (Student-Newman-Keuls test) to locate the significant difference indicated with ANOVA. A value of P<0.05 was considered statistically significant.

Results

Hyperglycemic Clamp

Control Subjects

During the clamp (Figure 1), plasma glucose stabilized at 15 mmol/L with oscillations not exceeding 5% of the prefixed value. Labile HbA1c levels increased from the value of 0.15±0.03% to 1.1±0.1% (P<0.01) at 5 hours; stable HbA1c levels did not show any significant change during the clamp. Insulin secretion in response to glucose was completely suppressed during octreotide infusion: Circulating insulin levels remained below 70 pmol/L during the study. Plasma IL-6 levels rose from a basal value of 2.0±0.7 pg/mL to a
peak of 3.1±0.9 pg/mL at 1 hour \((P<0.01)\) and returned to basal level at 3 hours (Figure 2). Fasting plasma TNF-\(\alpha\) levels were 3.3±1.2 pg/mL; they peaked at 1 hour (4.9±1.4 pg/mL, \(P<0.01\)), and returned to baseline at 3 hours. Plasma IL-18 levels rose from a basal value of 116±28 pg/mL to a peak of 140±31 pg/mL at 2 hours \((P<0.01)\) and returned to basal levels at 3 hours (110±26 pg/mL).

**IGT Subjects**

Plasma glucose, insulin, and labile HbA\(_1\) levels during the clamp were not significantly different from values obtained in control subjects. Fasting plasma IL-6 and TNF-\(\alpha\) levels were higher than those in control subjects (Table). The increase in plasma cytokine levels during the clamping lasted longer than in control subjects (4 hours versus 2 hours, respectively, \(P<0.01\)) and returned to basal levels at 5 hours (Figure 2). The infusion of octreotide alone in 5 control and 5 IGT subjects did not cause any significant variations in the plasma concentration of IL-6, TNF-\(\alpha\), and IL-18 levels (data not shown).

**Glucose Pulses**

**Control Subjects**

The fasting concentrations of plasma glucose and insulin were not significantly different from the corresponding values obtained in the clamp study. Plasma glucose peaked between 3 and 5 minutes after each pulse and returned to basal values before the subsequent glucose injection (Figure 1). Both first-phase (0 to 10 minutes) and second-phase (10 to 60 minutes) insulin secretion were completely suppressed by octreotide. The increase in labile HbA\(_1\) level during the study (1.1±0.2\%) was not different from that obtained in the clamp study (0.98±0.17\%). The fasting concentrations of IL-6 (1.9±0.7 pg/mL), TNF-\(\alpha\) (3.3±1.2 pg/mL), and IL-18 (112±28 pg/mL) were not significantly different from those obtained during the clamping. Plasma IL-6 levels peaked at 1 hour after the first glucose pulse and returned to baseline at 2 hours (Figure 3). Values obtained at 3, 4, and 5 hours were significantly \((P<0.05)\) higher than the corresponding values observed during the clamping. A similar trend of response was also observed for TNF-\(\alpha\) and IL-18: For both, the greatest peak occurred 60 minutes after the third glucose pulse, which was significantly greater than the baseline value \((P<0.01)\) and the corresponding 5-hour value obtained in the clamp study \((P<0.02)\).

**IGT Subjects**

Plasma glucose peaks, insulin, and labile HbA\(_1\) levels after glucose injections were not significantly different from values obtained in control subjects. Fasting cytokine levels were not significantly different from those obtained during the clamping. However, cytokine peaks after the glucose pulses were higher \((P<0.05)\) than the corresponding values obtained in control subjects and did not return to baseline by 2 hours (Figure 3).

**Glutathione Study**

The clinical and metabolic characteristics of the 10 control subjects participating in this study were not significantly different from the other 10. In particular, their age was 34.5±3.9 years and body mass index was 24.3±1.2 kg/m\(^2\), and there was no difference in the basal levels of plasma.
glucose (5.2±0.5 mmol/L), insulin (64±16 pmol/L), IL-6 (1.8±0.8 pg/mL), TNF-α (3.4±1.2 pg/mL), and IL-18 (115±25 pg/mL). During glutathione infusion, the 3 consecutive glucose pulses produced plasma glucose levels not significantly different from those observed without glutathione; plasma insulin levels were fully suppressed by octreotide. On the other hand, plasma IL-6, TNF-α, and IL-18 concentrations did not show any significant change from baseline after the three consecutive glucose pulses (Figure 4).

Similarly, there was no significant change from baseline in plasma cytokine concentrations during glutathione infusion in 8 IGT subjects receiving the 3 glucose pulses (Figure 4).

**Discussion**

The novel findings of the present study were that (1) acute hyperglycemia in control and in IGT subjects induces an increase in plasma IL-6, TNF-α, and IL-18 concentrations; (2) the effect of sustained hyperglycemia is reproduced by transient oscillations in plasma glucose and is amplified by the IGT status; and (3) the antioxidant glutathione completely prevents the rise in plasma cytokines induced by hyperglycemia. These results indicate that hyperglycemic spikes affect cytokine concentrations more than continuous hyperglycemia, at least in the short term, and suggest that an oxidative mechanism mediates the effect of hyperglycemia.

To our knowledge, this is the first demonstration that acute hyperglycemia affects the concentration of plasma cytokines in humans. In vitro studies using supraphysiological glucose concentration (>22 mmol/L) have reported an increase in TNF-α and IL-6 secretion from healthy human mononuclear cells. Furthermore, increased synthesis of TNF-α has been reported both in primary cultures of rat uterine cells cultured in vitro with increasing concentrations of glucose and in placental tissue explants from women with gestational diabetes incubated with high glucose (25 mmol/L). There is also evidence that IL-6 production by human monocytes isolated from healthy volunteers is enhanced during 24-hour incubation in high-glucose medium.

Previous studies have demonstrated increased circulating levels of TNF-α and IL-6 in diabetic patients, with a significant correlation of IL-6 levels with HbA1c, progression of diabetic nephropathy, and amelioration of glycemic control after treatment. In a more general sense, an augmented acute-phase response may be a mechanism that explains many of the clinical and biochemical features of type 2 diabetes and its complications. For example, TNF-α can impair insulin receptor signaling in adipose tissue and skeletal muscle, thereby decreasing insulin-stimulated glucose uptake and promoting insulin resistance associated with obesity and type 2 diabetes. Administration of human recombinant IL-6 in humans has been shown to induce hyperglycemia and compensatory hyperinsulinemia, two biological markers of insulin resistance. Moreover, subjects homozygous for the C allele of the IL-6 gene present lower levels of plasma IL-6 and an increased insulin sensitivity index than do carriers of the G allele. Lastly, elevated levels of IL-6 predict the development of type 2 diabetes in healthy women.

The TNF-α and IL-6 found in plasma are likely produced by various tissues, including activated leukocytes, adipocytes, and endothelial cells. Because the increased circulating cyto-
kine levels found in diabetes seem to originate from noncirculating cells, and given the prompt increase of plasma cytokine levels after acute hyperglycemia in the present study, likely candidates for its production may be adipocytes and endothelial cells. Further support for this hypothesis comes from the evidence that adipocytes secrete ≈25% of in vivo systemic IL-6 concentration and that high glucose increases the secretion of IL-1 from cultured human aortic endothelial cells.

We could not find any study that assessed the circulating levels of IL-18 in diabetes or the effect of high glucose on IL-18-producing cells in vitro. IL-18 is a pleiotropic cytokine acting in both acquired and innate immunity and might be involved in atherosclerosis. Beside acting as a proinflammatory cytokine by inducing the expression of adhesion molecules, IL-18 is also able to stimulate the production of granulocyte-macrophage colony-stimulating factor, TNF-α, and inducible nitric oxide synthase by mononuclear and mesenchymal cells. In human atheroma in situ, IL-18 signaling evokes effectors involved in atherogenesis, such as IL-6, intracellular adhesion molecule-1, and matrix metalloproteinases 1, 9, and 13; moreover, the recent demonstration of significantly higher levels of IL-18 mRNA in symptomatic plaques than in asymptomatic plaques suggests a major role in atherosclerotic plaque destabilization leading to acute ischemic syndrome. Following this line of thought, it might be speculated that the detrimental effect of stress hyperglycemia in acute coronary syndromes might also be due to the effect of acute hyperglycemia to increase circulating IL-18 levels.

Epidemiological studies published in recent years suggest that postprandial blood glucose might be an independent risk factor of cardiovascular disease and that postprandial hyperglycemia in diabetic subjects is a more powerful marker of cardiovascular disease risk than is fasting hyperglycemia. In this context, hyperglycemic spikes might be seen as surrogate markers of oscillations of plasma glucose levels after meals. We have shown that cytokine levels are affected more by oscillatory than continuous hyperglycemia and that the IGT status amplifies the phenomenon. The molecular mechanisms triggered by intermittent hyperglycemia are not known. However, some metabolic variations induced by sustained hyperglycemia might change or feed back regulatory cell controls, partially counteracting the glucose effects. Intermittent exposure to hyperglycemia might reduce such adaptation, causing more pronounced toxicity. Consistent with this, in human umbilical vein endothelial cells in culture, apoptosis is enhanced in response to intermittent, rather than continuous high glucose concentrations. The different pattern of cytokine secretion after hyperglycemic spikes might have clinical significance because prospective epidemiological studies have found increased vascular risk in association with increased levels of cytokines such as IL-6 and TNF-α.

Another finding of the present study was that glutathione, a powerful antioxidant, completely prevented cytokine increase induced by oscillatory hyperglycemia in healthy humans. Hyperglycemia-induced oxidative stress, along with soluble advanced glycation end products and products of lipid peroxidation, possibly serves as a key activator of upstream kinases, leading to induction of inflammatory gene expression. In cultured mononuclear cells incubated with high glucose (15 mmol/L for 18 hours) a dramatic increase in the release of TNF-α has been reported to be mediated by reactive oxygen species via activation of transcription factors nuclear factor-κB (NF-κB) and activating protein-1. Interestingly enough, IκB kinase β repression (leading to increased NF-κB deactivation) protects against the development of insulin resistance during high-fat feeding and in ob/ob mice.

The present study introduces an additional aspect of how hyperglycemia might contribute to early stages of atherogenesis and also favor cardiovascular death in myocardial infarction patients: Acute hyperglycemia increases circulating cytokine concentrations, which have been implicated in insulin resistance (TNF-α, IL-6), plaque destabilization (IL-18), and future cardiovascular events (IL-6). Although the relevance of these acute changes in plasma cytokines to the chronic vascular complications of diabetes is, at present, speculative, an increased oxidative stress seems a likely mechanism linking acute hyperglycemia to cardiovascular diabetic complications via an increased cytokine secretion.

References
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