Elevated Blood Interleukin-6 Levels in Hyperketonemic Type 1 Diabetic Patients and Secretion by Acetoacetate-Treated Cultured U937 Monocytes

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OBJECTIVE — Diabetic patients have elevated blood levels of interleukin-6 (IL-6), which is known to increase inflammation and the development of vascular disease and atherosclerosis. This study examined the hypothesis that ketosis increases the circulating levels of IL-6 in type 1 diabetic patients as well as the secretion of IL-6 in vitro in a cell culture model using U937 monocytes.

RESEARCH DESIGN AND METHODS — Fasting blood was obtained from type 1 diabetic patients and healthy siblings. To examine the effect of ketosis, U937 monocytes were cultured with ketone bodies (acetoacetate [AA], β-hydroxybutyrate [BHB]) in the presence or absence of high glucose levels in the medium at 37°C for 24 h. IL-6 was determined by the sandwich enzyme-linked immunosorbent assay method, and intracellular reactive oxygen species (ROS) generation was detected using dihydroethidium dye.

RESULTS — The blood level of IL-6 was higher in hyperketonemic (HK) diabetic patients than in normoketonemic (NK) diabetic patients ($P < 0.05$) and normal control subjects ($P < 0.05$). There was a significant correlation between ketosis and IL-6 levels ($r = 0.36$, $P < 0.04$, $n = 34$) in the blood of diabetic patients. Cell culture studies found that exogenous addition of the ketone body AA, but not BHB, increases IL-6 secretion and ROS generation in U937 cells. *N*-acetylcysteine (NAC) prevented the IL-6 secretion in acetoacetate-treated U937 monocytes.

CONCLUSIONS — This study demonstrates that hyperketonemia increases IL-6 levels in the blood of type 1 diabetic patients and that NAC can inhibit IL-6 secretion by U937 monocytic cells cultured in a ketotic medium.

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Interleukin-6 (IL-6), which is secreted by macrophages, lymphocytes, and other cells (1), is an important cytokine that can initiate events leading to atherogenesis by induction of adhesion molecules, monocyte-endothelial interactions, and inflammation injury (1–5). Anti–IL-6 therapy significantly prevents the inflammatory process in mice (6). The role of IL-6 in vascular inflammation has also been shown using IL-6 knockout mice that exhibit resistance to splanchnic artery occlusion shock (6), and in studies (7) that show increased levels of lipid peroxidation and inflammation in mice that overexpress IL-6. This suggests that elevated blood levels of IL-6 are associated with the development of vascular inflammation and atherosclerosis (1,2).

IL-6 levels in blood are higher or similar in diabetic patients compared with normal subjects (4,8–10). Cell culture studies have shown that high glucose concentrations can increase the IL-6 secretion in cultured monocytes (4,11,12). In addition to hyperglycemia, type 1 diabetic patients frequently experience ketosis (hyperketonemia) from excessive fat breakdown because body fuel is derived mainly from fat when the body is in a state of insulin deficiency (13). The blood concentration of ketone bodies (acetoacetate [AA], β-hydroxybutyrate [BHB]) may reach 10 mmol/l in patients with severe ketosis, as compared with levels of <0.5 mmol/l in normal individuals (13–15). The immediate concern in ketotic patients is acidosis and dehydration. Current standards of clinical practice do not allow an even milder degree of ketosis in diabetic patients (14–16). Nevertheless, ketonemia levels of 1–2 mmol/l (1–2 μmol/ml) are frequently seen in diabetic patients, even at the time of routine check-up visits to the clinic (15). It is known that diabetic subjects with frequent episodes of ketosis experience an increased incidence of vascular disease, morbidity, and mortality (15,16). However, the underlying mechanisms by which ketosis promotes vascular disease in type 1 diabetic patients are unclear. No study has been performed on the effects of ketosis on blood levels of IL-6 in diabetic patients or on IL-6 secretion by monocytes in a cell culture model.

This study examined the hypothesis that ketosis increases the IL-6 secretion in a cell culture model using U937 monocytes and in type 1 diabetic patients. Our data show that hyperketonemia is associated with increased IL-6 level in the blood of type 1 diabetic patients in vivo and that

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Abbreviations: AA, acetoacetate; AKB, α-ketobutyric acid; BHB, β-hydroxybutyrate; HK, hyperketonemic; IL-6, interleukin-6; NAC, *N*-acetylcysteine; NK, normoketonemic; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

the ketone body AA, but not BHB, stimulates the secretion of IL-6 in cultured U937 monocytic cells.

RESEARCH DESIGN AND METHODS

Diabetic patients and normal volunteers. Written informed consent was obtained from all subjects in accordance with the protocol approved by the Institutional Review Board for the Protection of Human Research Subjects. Blood from patients and healthy siblings was drawn after an overnight fast. Blood samples were collected into precooled tubes with EDTA kept in an ice bucket. The EDTA blood was centrifuged and the clear plasma saved for AA, BHB, and IL-6 assays. All analyses were performed immediately after blood collection. All patients were type 1 diabetic children and age-matched normal siblings. Patients with plasma AA levels ≤0.2 μmol/ml were considered normoketonemic (NK) and those with AA levels >0.2 were considered hyperketonemic (HK).

Human promonocytic cell line. The U937 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained at 37°C in RPMI-1640 medium containing 7 mmol/l glucose, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 12 mmol/l sodium bicarbonate, 12 mmol/l HEPES, and 2 mmol/l glutamine in a humidified atmosphere containing 5% (vol/vol) CO_2. For treatments, cells were washed once in plain RPMI-1640 before being suspended in fresh medium (complete) containing serum and other supplements (17).

Treatment with AA or BHB in normal or high glucose medium. U937 monocytes (one million cells/ml) were treated with AA or BHB (0–3 mmol/l). Treatments included use of both normal-glucose (7 mmol/l) and high-glucose medium (30 mmol/l), along with AA or BHB. For IL-6 secretion studies, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) and treatments were carried out at 37°C for 24 h. All experiments were repeated at least three times. α-Ketobutyric acid (AKB), a ketone not present in diabetic blood, served as an inert ketone control for AA and BHB. Deoxyglucose was used as an osmolarity control.

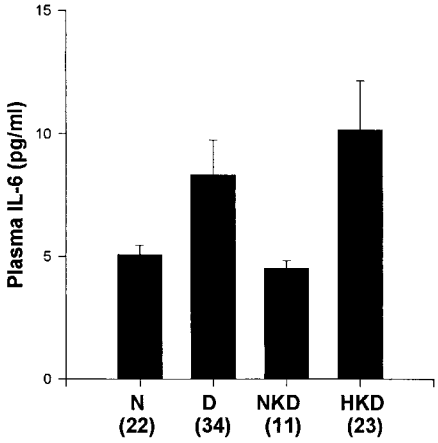

Figure 1—*Plasma IL-6 levels in NK and HK diabetic patients (D) and age-matched normal control subjects (N). Data are means ± SE. P < 0.05 for N vs. HK patients and NK vs. HK patients.*

AA, BHB, ROS, and IL-6 measurements. Plasma levels of AA and BHB were determined by enzymatic methods (18,19). Intracellular reactive oxygen species (ROS) generation was detected using dihydroethidium dye (Molecular Probes). When dye is cleaved by ROS, the fluorescent byproduct ethidium is produced, which is detected using a flow cytometer (20). IL-6 levels in the supernatant of treated cells and in the plasma of patients and normal subjects were determined by the sandwich enzyme-linked immunosorbent assay method using a commercially available kit from Neogen (Lexington, KY). All appropriate controls and standards as specified by the manufacturer were used; the data are expressed as picograms IL-6 secreted per one million cells or per milliliter plasma.

Information on age, duration of diabetes, and clinical laboratory tests, such as HbA_{1c},were collected from patients' medical records in the diabetes clinic. In the cytokine assay, control plasma samples were analyzed each time to check the variation from plate to plate on different days of analyses. The assays were repeated if the variation in control plasma value from day to day was >7%. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise mentioned. Data between different groups were analyzed statistically using one-way ANOVA with Sigma Plot and Sigma Stat statistical software (SPSS, Chicago, IL). For patient's data, Kruskal-Wallis one-way ANOVA on ranks and multiple comparison procedures (Dunn's Method) was used to analyze differences between groups (Fig. 1) and Spearman rank-order correlation was used to analyze relationship. For data given in Figs. 2–4 (in vitro studies), one-way ANOVA and multiple comparisons versus control group (Bonferroni *t* test) were performed. A $P < 0.05$ was considered significant.

RESULTS — Figure 1 illustrates that IL-6 levels are higher but not statistically significant in diabetic patients compared with age-matched normal subjects. When diabetic patients were divided into NK and HK groups, HK patients had significantly higher levels ($P < 0.05$) of IL-6 compared with those of NK patients or normal control subjects. However, there

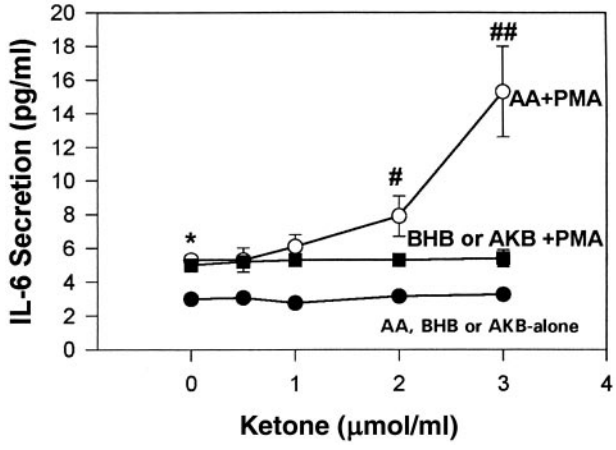

Figure 2—*Effect of different concentrations of AA, BHB, and AKB on IL-6 secretion by cultured U937 monocytes. Data are means ± SE of four experiments. Differences between values (* [control] versus # and * versus ##) among AA + PMA–treated cells are significant (P < 0.05).*

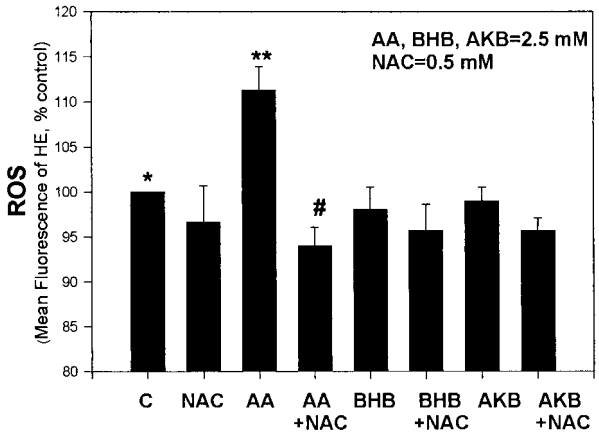

Figure 3—*Effect of AA, BHB, AKB, and NAC on ROS production in U937 monocytes. Data are means ± SE of four experiments. Differences between * (control) versus ** and * versus # are significant* ($P < 0.05$).

was no difference in the levels of IL-6 in NK patients compared with those of age-matched normal subjects. Table 1 shows that there was no difference in duration of diabetes or age between NK and HK patients or in age between normal and diabetic groups. HbA_{1c} levels between NK and HK patients were not significantly different. This suggests that ketosis is associated with the elevated IL-6 levels in diabetic patients. This study also found a significant relationship between the ketosis (as determined by AA level) and IL-6 levels ($r = 0.36$, $P < 0.04$, $n = 34$) in the blood of type 1 diabetic patients. However, the relationship between IL-6 levels and either blood glucose (0.31, $P = 0.07$) or HbA_{1c} (0.13, $P = 0.45$) levels was not significant.

To examine the biochemical mechanisms leading to elevated IL-6 levels in HK patients, we studied IL-6 secretion in a U937 monocytic cell line cultured with elevated levels of AA or BHB with and without high glucose levels in the culture medium. Figure 2 shows that AA, BHB, or AKB alone does not have any effect on IL-6 secretion in unstimulated monocytes. However, in PMA-activated monocytes, treatment with AA, but not BHB or AKB, caused a concentration-dependent increase in IL-6 secretion. Figure 3 illustrates a modest but significant increase ($P < 0.05$) in ROS production in AA-treated compared with control monocytes. This increase in ROS production was prevented in the presence of NAC. BHB and AKB treatments did not cause an increase in ROS production. Figure 4 illustrates that the effect of AA on IL-6 secretion was pronounced in the presence of high glucose. However, BHB did not influence the IL-6 secretion caused by high glucose. Deoxyglucose used as an osmolarity control also did not have any effect on IL-6 secretion (Fig. 4). Figure 4 also shows an inhibition in AA- and high-glucose–induced IL-6 secretion in the presence of NAC. This suggests that IL-6 secretion caused by elevated AA levels can be inhibited by NAC in an in vitro cell culture model.

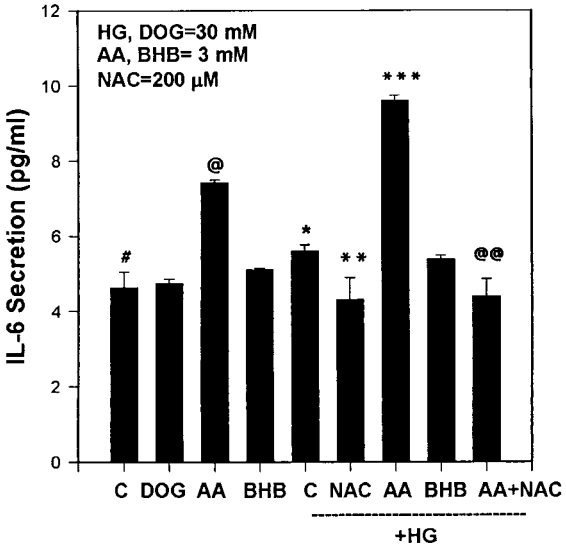

Figure 4—*Effect of high glucose, deoxyglucose, AA, BHB, and NAC on IL-6 secretion by activated monocytes. Data are means ± SE of three experiments. Differences between values marked # (control) versus @, * (control) versus **, * versus ***, and * versus @@ are significant* ($P < 0.05$). *DOG, deoxyglucose; HG, high glucose.*

CONCLUSIONS — Body cells are continuously generating ROS via the action of a variety of oxidases, such as xanthine oxidase, monoamine oxidase, NADPH oxidase, and urate oxidase, or by the auto-oxidation of many chemicals by molecular oxygen or mitochondrial respiration (21–23). In addition, oxidative stress in diabetes can arise from a variety of mechanisms. These mechanisms include excessive oxygen radical production as a result of the auto-oxidation of glucose (24), the activation of P-450–like activity by the glucose metabolite NADPH (25), glycated proteins (22,26) and the ketone body AA (27–30), depletion of NADH by the activation of aldose reductase (31), the ketosis associated increase in extramitochondrial oxidation of fatty acids and generation of hydrogen peroxide (21), and glycation of antioxidative enzymes, which limits their capacity to detoxify oxygen radicals (32,33). Therefore, type 1 diabetic patients may experience oxidative stress from both hyperglycemia and ketosis (29,34).

The inhibition of superoxide radical generation prevents activation of protein kinase C, formation of advanced glycation end products, sorbitol accumulation, and nuclear factor-κB activation in high-glucose–treated cultured endothelial cells (31). Oxidants, such as hydrogen peroxide, have been previously shown to activate nuclear factor-κB and IL-6 in cultured monocytes and endothelial cells (12,23,35). On the other hand, hyperketonemia increases the oxidative stress (27–30). Thus, we hypothesize that ketosis increases the IL-6 secretion in a cell culture model using U937 monocytes and in type 1 diabetic patients.

This study shows that IL-6 levels are higher in HK but not NK patients in comparison with normal subjects. The diabetic patients in this study were children who did not show any signs of clinical

Table 1—*Age, duration of diabetes, HbA_{1c}, and ketosis level in NK and HK diabetic patients, all diabetic patients, and normal subjects*

	Normal subjects	All diabetic patients	NK diabetic patients	HK diabetic patients
n	22	34	11	23
Age (years)	11 ± 1	13 ± 1	12 ± 1	13 ± 1
Duration of diabetes (years)	—	5 ± 1	5 ± 1	6 ± 1
Glucose (mmol/l)	5.03 ± 0.14*	8.31 ± 0.3†	8.62 ± 0.5	8.2 ± 0.3
HbA_{1c} (%)	5.0 ± 0.4*	10.8 ± 1.0†	10.3 ± 1.5	11 ± 1.3
AA (mmol/l)	0.19 ± 0.02*	0.25 ± 0.02†	0.17 ± 0.01‡	0.30 ± 0.03§
BHB (mmol/l)	0.10 ± 0.03*	0.15 ± 0.04†	0.07 ± 0.02‡	0.19 ± 0.06§
Total ketones	0.29 ± 0.04*	0.41 ± 0.06†	0.23 ± 0.03‡	0.49 ± 0.08§

Data are means ± SE. Differences in values between * versus †($P < 0.05$) and between ‡ versus § are significant ($P < 0.02$).

complications. Our data, together with those from previous studies on IL-6 levels in newly diagnosed diabetic children (8,9), suggest that the elevated levels of IL-6 in diabetic patients are not due to the complications associated with diabetes. Our study shows that the IL-6 secretion in activated monocytes was stimulated by both AA and high glucose, separately as well as when used together, whereas BHB did not have any effect on IL-6 secretion. Similarly, AA can generate ROS, whereas BHB does not, which suggests that ROS may be involved in the increased IL-6 secretion in AA-treated monocytes. Indeed, the effect of AA and high glucose on IL-6 secretion was prevented by the antioxidant NAC. Taken together, these studies suggest a potential role for ROS generation in the increased IL-6 secretion in the AA-treated monocytes. However, it is not known whether elevated levels of IL-6 play a role in the increased oxidative stress levels observed in HK patients because overexpression of IL-6 can also increase lipid peroxidation levels in mice (7). Whether ketosis has any effect on IL-6 receptors, which are known to influence the circulating IL-6 levels, is not known.

In conclusion, this study demonstrates for the first time that ketosis can significantly increase the effect of hyperglycemia on IL-6 secretion by U937 monocytes in a cell culture model and in type 1 diabetic patients. Whether ketosis can increase induction of adhesion molecules, thereby increasing monocyte-endothelial cell adhesion and the development of vascular disease and atherosclerosis, is not known (36). The evidence that the antioxidant NAC can prevent the secretion of IL-6 in AA-treated cultured monocytes needs to be explored at the clinical level to determine whether dietary supplementation in humans can prevent or delay the excess vascular disease observed among the diabetic patient population.

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