Hyperketonemia Can Increase Lipid Peroxidation and Lower Glutathione Levels in Human Erythrocytes In Vitro and in Type 1 Diabetic Patients

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Recent studies have suggested that elevated cellular lipid peroxidation may play a role in the development of cellular dysfunction and other complications of diabetes. People with type 1 diabetes frequently encounter elevated levels of the ketone bodies acetoacetate (AA), β-hydroxybutyrate (BHB), and acetone (ACE). This study was undertaken to test the hypothesis that ketosis might increase lipid peroxidation and lower glutathione (GSH) levels of red blood cells (RBCs) in diabetic patients. This study demonstrates that incubation of AA with normal RBCs in phosphate-buffered saline (37°C for 24 h) resulted in marked GSH depletion, oxidized glutathione accumulation, hydroxyl radical generation, and in-creased membrane lipid peroxidation. Increases in oxygen radicals and lipid peroxidation and depletion of GSH in RBCs were not observed with BHB or ACE treatments. Similarly, there was a significant generation of superoxide ion radicals even in a cell-free buffer solution of AA, but not in that of BHB. The presence of BHB together with AA did not influence the capacity of AA to generate oxygen radicals in a cell-free solution or the increase in lipid peroxidation of RBCs incubated with AA. The antioxidants vitamin E and N-acetylcysteine (NAC) blocked increase in lipid peroxidation in AA-treated RBCs. To examine the effects of ketone bodies in vivo, studies were performed that showed a significant decrease in GSH and an increase in lipid peroxidation levels in RBCs of hyperketonemic diabetic patients, but not in normoketonemic type 1 diabetic patients, when compared with age-matched normal subjects. This study demonstrates that elevated levels of the ketone body AA can increase lipid peroxidation and lower GSH levels of RBCs in people with type 1 diabetes. *Diabetes* 48:1850–1855, 1999

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AA, acetoacetate; ACE, acetone; BHB, β-hydroxybutyrate; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; NAC, N-acetylcysteine; NBT, nitroblue tetrazolium; RBC, red blood cell.

Various factors, including hyperglycemia, glycation of proteins, and accumulation of sorbitol, have been proposed to contribute to the pathogenesis of cellular dysfunction leading to the vascular complications of diabetes (1–3). Red blood cells (RBCs) and other cells of diabetic animals and patients have elevated levels of lipid peroxidation products (4–14). Recent studies showing a decrease in the occurrence of complications after supplementation with different antioxidants in a diabetic animal model provide evidence for the role of lipid peroxidation in the development of diabetic complications (15–22).

In uncontrolled type 1 diabetes, where body fuel is mainly derived from fat, the level of ketone bodies begins to rise in the blood (ketonemia) (23). The blood concentration of ketone bodies may reach 10 mmol/l in diabetic patients with severe ketosis, versus <0.5 mmol/l in normal people (24,25). It is known that diabetic patients with frequent episodes of ketosis have increased incidence of vascular disease, neuropathy, morbidity, and mortality (26). The level of lipid peroxidation in blood is higher in diabetic patients with vascular disease (27,28). However, it is not known whether ketone bodies play any direct role in the increased lipid peroxidation and lower glutathione (GSH) level in RBCs, which, in turn, may contribute to the development of cellular dysfunction.

This study was undertaken to test the hypothesis that frequent episodes of hyperketonemia can increase lipid peroxidation and lower GSH levels in RBCs in type 1 diabetic patients. The specific objective of this study was to examine lipid peroxidation and GSH levels of RBCs after in vitro treatment with ketone bodies, acetoacetate (AA), β-hydroxybutyrate (BHB), and acetone (ACE), and in RBCs from type 1 diabetic patients with and without hyperketonemia.

RESEARCH DESIGN AND METHODS

Blood was collected into tubes containing EDTA from normal human volunteers according to a protocol approved by the Institutional Human Experiments Review Committee. The EDTA blood was centrifuged, and the clear plasma and the buffy coat were discarded. The RBC suspension was filtered through cotton wool to remove any leftover leukocytes. The resultant cells were washed with a cold 0.15 mol/l sodium chloride solution three times after a 1:10 dilution.

In vitro treatment with ketone bodies, vitamin E, and NAC. Washed RBCs were suspended to 15% hematocrit in phosphate-buffered saline (29) containing 6 mmol/l of glucose. Aliquots of cell suspension were placed in Erlenmeyer flasks, after which a freshly prepared stock solution of AA, BHB, or ACE was added to the flasks. Concentrations are expressed in terms of the total cell suspension. The contents of the flasks were incubated in a shaking water bath at 37°C for

TABLE 1
Effect of ketone bodies on GSH, GSSG, lipid peroxidation, and hydroxyl radicals generation in RBCs

Treatment	None	AA (20 µmol/ml)	BHB (20 µmol/ml)	ACE (20 µmol/ml)
Hydroxylated benzoic acid (nmol/g Hb)	7 ± 2*	19 ± 2†	8 ± 3	9 ± 3
GSH (µmol/g Hb)	4.78 ± 0.30*	1.70 ± 0.17†	4.70 ± 0.24	5.41 ± 0.39
GSSG (µmol/g Hb)	0.59 ± 0.04*	1.38 ± 0.05†	0.61 ± 0.04	0.64 ± 0.07
MDA (nmol)				
Per packed cell volume	2.01 ± 0.09*	3.33 ± 0.24†	2.06 ± 0.09	2.17 ± 0.15
Per gram Hb	4.4 ± 0.6*	7.5 ± 0.7†	4.9 ± 0.3	4.4 ± 0.6

Data are means ± SE of five experiments. Differences in the values marked * and † are significant ($P < 0.01$). Note a significant decrease in GSH and increase in GSSG, MDA, and hydroxyl radicals generation in AA-treated, but not in BHB- or ACE-treated, RBCs.

24 h. In certain experiments, the RBC suspension was preincubated with NAC or vitamin E for 30 min before the addition of AA to the suspension. In the case of vitamin E, D-α-tocopherol in alcohol was layered at the bottom of a 25-ml Erlenmeyer flask. Alcohol was then dried with nitrogen before RBC suspension was added to the flask. Percent hemolysis was <1% in all incubations. Treated RBCs were washed two times after a 1:10 dilution with 0.15 mol/l NaCl before biochemical analyses. All incubations contained 10 µl of penn-strept/ml of cell suspension to vitiate any microbial growth during the overnight incubations. The working solution of penn-strept contained 300 mg of pencillin G and 500 mg of streptomycin in 10 ml of buffer.

AA, BHB, and superoxide anion radical measurements. Levels of AA were determined by the method of Artuch et al. (30), and levels of BHB by the method of Koch and Feldbruegge (31). The generation of superoxide anion radicals in the aerobic reaction mixture was determined using the nitroblue tetrazolium (NBT) reduction method (32). This method monitors reduction of NBT by the superoxide anion radical at 540 nm (32). In this case, the change in absorbance of NBT by ketone bodies was calculated after subtracting the absorbance of NBT in the reaction buffer without and with the ketone bodies. All reactions were carried out at 37°C under aerobic conditions. The volume of reactant reaction mixture is given in the legend to Fig. 2.

GSH, oxidized glutathione, hydroxyl radicals, and lipid peroxidation measurements in treated RBCs. Levels of GSH and oxidized glutathione (GSSG) were determined after high-performance liquid chromatography (HPLC) separation, as described by Reed et al. (33). Hydroxyl radical generation was determined by the nonenzymatic hydroxylation of sodium benzoate. RBCs were incubated in the presence of sodium benzoate for 24 h as described previously (34), except that ketone bodies were used instead of ascorbic acid. Separation of the products of benzoate hydroxylation (3-hydroxybenzoate and 4-hydroxybenzoate) was accomplished using HPLC with an ultraviolet/vis detector at 280 nm and a column and solvent system as described (35). Membrane lipid peroxidation was determined by the HPLC of malondialdehyde (MDA), an end product of fatty acid peroxidation, and of the thiobarbituric acid complex using ion exclusion and a reverse-phase Shodex KC-811 column (Waters, Milford, MA) with the detector set at 532 nm (36). The packed cell volume of washed RBCs was determined using an Autocrit centrifuge (Beckton Dickinson, Sparks, MD).

Diabetic children and their healthy siblings. Informed written consent of all patients was obtained in accordance with the protocol approved by the Institutional Human Experiments Review Committee. Diabetic patients with any other diseases were excluded from this study. Diabetic patients and their healthy siblings who agreed to participate in this study were asked to come to the clinic after fasting overnight and before taking any insulin. They were told to bring their insulin and syringes for use after drawing blood. Blood was collected into tubes with EDTA. All analyses were performed immediately after blood collection. All patients were insulin dependent (type 1 diabetes). Diabetic subjects with plasma AA levels of 0.2 µmol/ml were considered normoketonemic and those with AA levels >0.2 µmol/ml were considered hyperketonemic. Fasting blood HbA_1 levels were determined by Glyc-affinity columns (Isolab, Akron, OH), and glucose levels in the clinical laboratory at the school.

All chemicals were purchased from Sigma (St. Louis, MO), unless otherwise mentioned. AA was 99% pure as per the certificate of analyses provided by Sigma. The data were analyzed using the Kruskal-Wallis analysis of variance test with Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A P value of <0.05 was considered significant.

RESULTS

Table 1 shows a decrease in GSH levels and increases in GSSG, MDA, and hydroxyl radical generation in RBCs treated with AA, but not in RBCs treated with BHB or ACE. This suggests that AA, but not BHB and ACE, can generate oxygen radicals, oxidize cellular GSH, and induce accumulation of GSSG and MDA in RBCs. Figure 1 illustrates the effects of increasing concentrations of AA on GSH, GSSG, and MDA levels in RBCs. This suggests that elevated AA levels can cause GSH depletion and accumulation of GSSG and MDA in the RBC. The GSH depletion and MDA accumulation increased with increasing concentrations of AA. The increase in GSSG could not account for the GSH decrease because, unlike GSH, GSSG is permeable across the RBC membrane and thus, a portion of the GSSG is likely to have been lost in the buffer (supernatant).

The NBT reduction test originally reported by Beauchamp and Fridovich (37) is widely used as a fast and sensitive test for monitoring superoxide radical ion generation (33,37). Figure 2 illustrates the effect of AA and BHB on the reduction of NBT. There was a concentration-dependent reduction of NBT by AA. In comparison to AA, the generation of superoxide anion radicals by BHB was negligible. The level of superoxide radical generation by AA was similar in the absence or presence of BHB in the solution. The capacity of AA to generate superoxide ion radical was similar irrespective of whether phosphate, carbonate, or glycylglycine buffer was used in the experiments (data not given). Similarly, Table 2 shows that the level of lipid peroxidation and GSH deple-

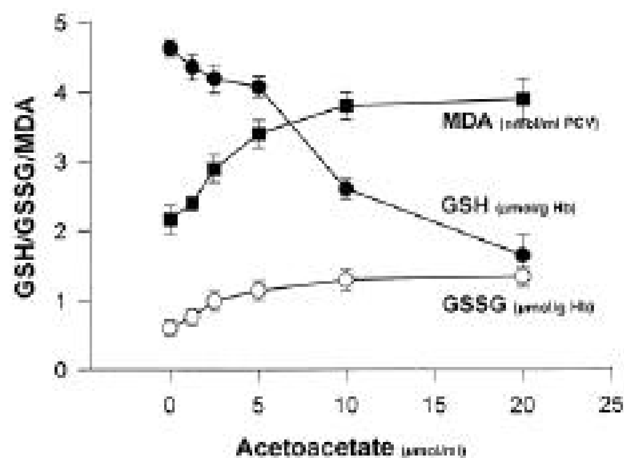

FIG. 1. Effect of different concentrations of AA treatment on GSH, GSSG, and MDA levels in RBCs. Values are means ± SE of five experiments. Note a significant ($P < 0.01$) decrease in GSH and increases in GSSG and MDA relative to control subjects with all concentrations of AA.

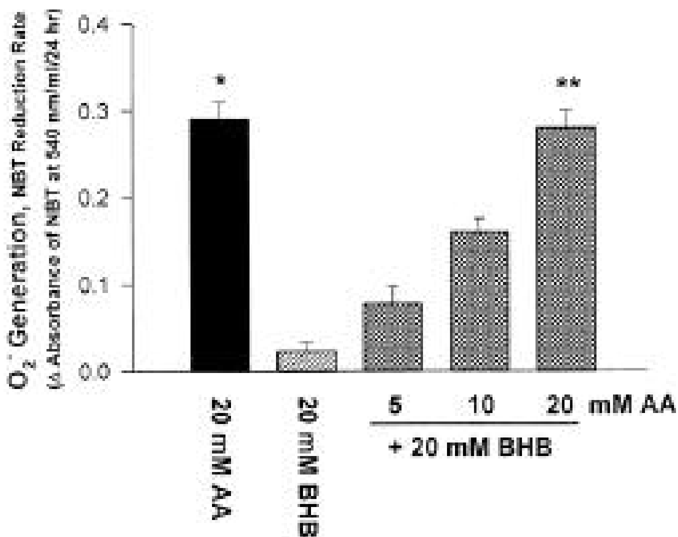

FIG. 2. Reduction of NBT by AA and BHB: effect of different concentrations of AA and BHB on superoxide anion radical (O_2^-) generation. Each value is the mean ± SE of four experiments. Generation of O_2^- was assessed by monitoring the reduction of NBT. Note an increase in the O_2^- generation with increasing concentrations of AA. Differences in values marked * and ** were not significant. The 1.5 ml of phosphate buffer contained 0.25 mmol/l NBT, 10 μm Fe^{++}, and different concentrations of AA or BHB. The mixture was incubated for 15 min at 37°C. Absorbance was read at 540 nm.

tion was similar in AA-treated RBCs in the absence or presence of BHB. Table 2 also shows that the pH of the incubation medium of AA- or BHB-treated RBC suspensions was similar to control subjects.

To determine whether there is any relationship between an increase in membrane lipid peroxidation and GSH depletion in RBCs, we treated RBCs with AA in the presence of NAC and vitamin E. GSH replenishment by NAC blocked GSH depletion and lowered the membrane lipid peroxidation in AA-treated RBCs (Table 3). Vitamin E did not affect GSH depletion, but it did block membrane lipid peroxidation in AA-treated RBCs. This suggests that GSH depletion may play a role in the increased membrane lipid peroxidation of AA-treated RBC.

There was no conversion of BHB to AA in BHB-treated, or conversion of AA to BHB in AA-treated, RBCs. This reconfirms that, unlike other tissues, RBCs do not have mitochondrial enzymes such as β-hydroxybutyrate dehydrogenase for the interconversion of AA to BHB. We did not measure acetone levels in our incubations, and because of the volatility of ACE, it is possible that the ACE concentrations present during the incubation were lower than those initially added.

To examine whether ketone bodies can deplete GSH and affect lipid peroxidation levels in vivo, we examined RBCs from type 1 diabetic patients. Diabetic subjects were divided into normoketonemic and hyperketonemic. There were higher levels of AA in hyperketonemic, but not in normoketonemic, diabetic patients in comparison to normal subjects (Table 4). Levels of age, fasting glucose, and HbA_1 were similar between normoketonemic and hyperketonemic diabetic subjects. Levels of GSH, however, were significantly lower, and levels of lipid peroxidation significantly higher (even after normalization of lipid peroxidation with total lipid) than control subjects in the RBCs of diabetic patients who had elevated ketone bodies, but not in diabetic patients with normal levels of ketone bodies. This suggests that elevated ketone body levels do influence the cellular GSH and lipid peroxidation levels in vivo as well.

DISCUSSION

A number of studies have proposed that elevated blood level of lipid peroxidation might be involved in cellular dysfunction, the thromboxane/prostacyclin imbalance, hypercoagulability, hypertension, and atherosclerosis (38–42). The ability of AA to generate superoxide anion radicals is a novel observation. Superoxide anion radicals can also form hydroxyl radicals in the presence of iron via a reaction called the Fenton reaction (18). These oxygen radicals exert their cytotoxic effect by causing peroxidation of membrane phospholipids and the resulting accumulation of peroxidation products such as MDA (18,19). These products have been known to cross-link membrane components and result in altered membrane permeability and lipid organization and cellular dysfunction (20–27).

This study has demonstrated increased levels of hydroxyl radicals and lipid peroxidation and a reduced level of GSH in AA-treated RBCs. GSH is known to scavenge free radicals and reactive oxygen intermediates (38). Thus, GSH depletion can further hamper cellular defenses against oxidative stress, resulting in cellular lipid peroxidation (38). The levels of membrane lipid peroxidation in AA-treated RBCs was blocked both by antioxidant vitamin E and by replenishment of cellular GSH by NAC. This suggests that an increase in the lipid peroxidation is mediated, at least in part, by GSH depletion in AA-treated RBCs. The in vitro studies also suggest that the presence of BHB together with AA does not influence the levels of lipid peroxidation and GSH depletion by AA in RBCs. There was some variation in the GSH and MDA values in apparently identical incubation conditions (Tables 1–3). This variation occurs because the RBCs of different volunteers were used for different sets of experi-

TABLE 2
Effect of BHB on membrane lipid peroxidation and GSH levels of AA-treated RBCs

	None	20 AA	20 BHB	5 AA	5 AA + 20 BHB	20 AA + 20 BHB
GSH (μmol/g Hb)	5.7 ± 0.3*	1.9 ± 0.2†	5.5 ± 0.2	4.5 ± 0.2‡	4.7 ± 0.2§	1.8 ± 0.2‖
MDA (nmol/g Hb)	4.3 ± 0.3*	6.4 ± 0.5†	4.5 ± 0.3	5.4 ± 0.2‡	5.4 ± 0.3§	6.6 ± 0.7‖
pH (of suspension)	7.26 ± 0.02	7.22 ± 0.02	7.24 ± 0.02	7.23 ± 0.03	7.24 ± 0.02	7.22 ± 0.02

Data are means ± SE of four experiments. 20 AA, 20 BHB, or 5 AA + 20 BHB are 20 mmol/l AA, 20 mmol/l BHB, or 5 mmol/l AA + 20 mmol/l BHB, and so on. Differences between * and † ($P < 0.01$) and between * and ‡ ($P < 0.05$) were significant. Differences between † and ‖ and between ‡ and § were not significant.

TABLE 3
Effect of vitamin E and GSH replenishment on membrane lipid peroxidation level of AA-treated RBCs

Treatment	None	NAC (1 μmol/ml)	Vitamin E (0.1 μmol/ml)	AA (20 μmol/ml)	AA + NAC	AA + E
GSH (μmol/g Hb)	6.0 ± 0.1*	6.8 ± 0.1	6.1 ± 0.2	2.3 ± 0.2†	4.2 ± 0.2‡	1.9 ± 0.2
MDA (nmol/ml cells)	2.0 ± 0.2*	2.0 ± 0.1	1.9 ± 0.2	3 ± 0.2†	2.3 ± 0.2‡	1.8 ± 0.1‡

Data are means ± SE of four experiments. Differences between * and † ($P < 0.01$) and between † and ‡ ($P < 0.01$) are significant. Note a significant increase in GSH after NAC addition and a significant decrease in MDA level after NAC and vitamin E addition during AA treatment of RBCs.

ments. Again, each set of experiments was repeated several times (n) using the RBCs of different normal volunteers. Because GSH and MDA levels vary from individual to individual, a variation in the mean GSH and MDA values of RBC in different tables is seen.

This study also demonstrates that the level of cellular oxidative damage, such as GSH depletion and lipid peroxidation, was significantly greater in diabetic subjects who had elevated levels of ketone bodies. The present in vitro and in vivo studies with RBCs from hyperketonemic diabetic subjects demonstrate that hyperketonemia is a risk factor in the elevated lipid peroxidation and GSH depletion in diabetic patients. The proposed mechanism by which AA can generate oxygen radicals and cause cellular lipid peroxidation may involve the formation of an ene-diol intermediate by the addition of $2H^+$ to two C=O groups in AA and its autoxidation that generates superoxide radicals. This can further undergo the Fenton reaction in the presence of Fe^{++} to form Fe^{+++} and hydroxyl radicals (35). Unlike β-hydroxybutyrate and acetone, the chemical structure of acetoacetate includes two keto groups. Whether this difference in chemical structure has anything to do with the potency of acetoacetate to generate oxygen radicals is not clear.

Some studies have reported no effect of diabetes on GSH levels (43), in contrast to other reports showing lower GSH levels in RBCs of diabetic patients compared with nondiabetic subjects (44–51). The present study suggests that differences in ketonemia may have contributed to this discrepancy in GSH level of RBCs in diabetes. One study suggests a role of decreased activity of γ-glutamylcysteine synthetase, a rate-limiting enzyme in GSH synthesis, in lowering GSH levels in the RBCs of diabetic patients (49). Whether ketone bodies can impair the activity of γ-glutamylcysteine synthetase is not known.

Diabetic patients in this study did not seem to have had severe episodes of ketosis because while their levels of AA were elevated, these levels were much lower than those known to occur in patients with coma and severe insulin deficiency (24,25) or that have been used in in vitro experiments. However, authors like to emphasize that, in patients, ketone bodies are constantly being excreted in the urine over time. Therefore, depending on the time elapsed, the measured blood levels are likely to be much lower than they might have been at the time of the ketotic episode. Second, RBCs survive in the circulation for a longer period and will reflect the accumulative effects of ketosis over the period of RBC life span, in contrast to only 24-h exposure in in vitro experiments. It is unlikely that other factors, such as altered blood glucose, bicarbonate, and insulin levels, account for the elevated lipid peroxidation levels in the RBCs of hyperketonemic diabetic subjects because blood was taken from overnight-fasted patients before the insulin injections. Second, the RBCs were washed before biochemical measurements, which removes cellular glucose. More importantly, similar results were obtained with in vitro studies in which other factors were controlled.

Hyperglycemia in type 1 and type 2 diabetes, in addition to other effects, can cause glycation of membrane proteins and increase cellular oxidative stress (2,29). Recent studies suggest that increased oxidative stress may promote the development of vascular disease and other diabetic complications (15–22). In addition, the lack of direct insulin effects on metabolic pathways due to insulin deficiency can influence cellular dysfunction in type 1 diabetes. Hyperketonemia or keto-

TABLE 4
GSH and MDA levels of RBCs from diabetic patients with and without elevated ketone bodies and age-matched normal subjects

	Normal subjects	Diabetic subjects with Normoketonemia	Diabetic subjects with Hyperketonemia
n	11	7	7
Age (years)	11.4 ± 1.3	13.2 ± 1.3	13.3 ± 0.6
Acetoacetate (μmol/ml)	0.13 ± 0.01*	0.15 ± 0.01*	0.71 ± 0.12†
β-Hydroxybutyrate (μmol/ml)	0.12 ± 0.06*	0.12 ± 0.04*	1.40 ± 0.66†
Glucose (μmol/ml)	4.9 ± 0.1*	10.0 ± 1.7†	13.1 ± 3.4†
HbA_1 (%)	5.9 ± 0.2*	13.2 ± 1.2†	11.6 ± 0.9†
GSH (μmol/g Hb)	6.40 ± 0.30*	5.88 ± 0.31‡	5.02 ± 0.21†§
MDA (nmol/g Hb)	3.7 ± 0.2*	3.9 ± 0.4	4.7 ± 0.2†
MDA (nmol/ml packed cell volume)	1.31 ± 0.05‡	1.35 ± 0.12	1.50 ± 0.06§
MDA (nmol/μmol lipid)	0.24 ± 0.01*	0.26 ± 0.02	0.30 ± 0.02†

Data are means ± SE. Differences between * and † ($P < 0.01$) and between ‡ and § ($P < 0.05$) are significant.

sis is mainly encountered in type 1 diabetic patients. The present study suggests that frequent episodes of ketosis can increase cellular oxidative stress in type 1 diabetes.

In conclusion, this study, for the first time, has demonstrated increased oxidative stress in RBCs exposed to elevated AA concentrations in vitro and in RBCs of hyperketonemic type 1 diabetic patients in whom excessive AA is produced endogenously in vivo. This suggests that ketosis is a risk factor in the development of cellular oxidative stress in diabetes.

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