Fetal Hemoglobin in Starvation Ketosis of Young Women

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Ketones can reactivate the production of fetal hemoglobin (HbF) in vitro and in vivo. A reactivation of HbF by ketones, which are generated during starvation, remains largely speculative. Therefore, we investigated HbF in 31 women with anorexia nervosa or bulimia, using both of these as models of intermittent starvation ketosis. For comparison, we also studied 42 female control subjects matched for age. β-Hydroxybutyrate levels were higher in patients than in controls (460 ± 90 *v* 110 ± 20 μmol/L; *P* < .0001). We correlated β-hydroxybutyrate, metabolic, and hematologic parameters with HbF. HbF was measured with high pressure liquid chromatography. The data were analyzed with logistic regression analysis. An elevated HbF fraction (>0.87%) was observed four times as often in patients than in controls (29% *v* 7%, *P* = .01). After adjustment for age, we found HbF elevations associated with β-hydroxybutyrate levels (*P* = .005). No other correlations between the various metabolic/hematologic parameters and HbF were significant. In conclusion, β-hydroxybutyrate generated in starvation is associated with increased levels of HbF. Thus, unrestrained lipolysis can produce β-hydroxybutyrate in sufficient quantities to induce a clinically measurable amount of HbF. These findings suggest that intermittent ketosis might also explain some increases of HbF in type 1 diabetes and pregnancy.

IN BOTH STARVATION and poorly controlled type 1 diabetes, low insulin levels result in augmented lipolysis and ketogenesis. Ketones, administered in pharmacologic doses, can reactivate the production of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) in adult patients.[1,2] During early childhood, the level of HbF normally decreases to less than 1% of the total Hb. However, increased levels of HbF are found in adult life not only in several inherited, eg, β-thalassemia, but also in acquired disorders, eg, type 1 diabetes,[3,4] and in pregnancy.[5] It is unknown whether ketones, which are generated from unrestrained lipolysis in the hypoinsulinemic state, could account for a clinically measurable increase in HbF. Therefore, we investigated HbF in young women with anorexia nervosa or bulimia, using both of these as models of intermittent starvation ketosis.[6,7]

MATERIALS AND METHODS

Subjects. Thirty-one women suffering from anorexia nervosa or bulimia and 42 female control subjects matched for age were studied (Table 1). The patients with anorexia nervosa (n = 10) or bulimia (n = 21) were investigated on the day of hospitalization, before they were started on an inpatient program at the Medical University of Lübeck. Diagnoses were made according to DSM-III-R criteria.[8] Control subjects were chosen from among students attending a school of economics. Age was 25 ± 1 years (mean ± SEM) in both groups (range, 18 to 34 years in the patients and 15 to 39 years in the control subjects). None of the study subjects had a history of diabetes mellitus, acute blood loss within the last 8 weeks, present pregnancy, cancer, or hematologic disease, as these are conditions that might affect the production of HbF.[9] None of the study subjects reported past or present alcohol or drug abuse. The control subjects had no history or current symptoms of eating disorders. Before inclusion in the study, every person signed a consent form which had been approved by the local ethics commission (for patients <18 years of age, her parents would sign).

Analytical methods. Blood samples were drawn after a 12-hour overnight fast for measurements of β-hydroxybutyrate, HbF, and other metabolic and hematologic parameters. Serum β-hydroxybutyrate was measured with a photometric method using a digital photometer 6114 S (Eppendorf Netheler Hinz, Hamburg, Germany). The β-hydroxybutyrate intra-assay coefficient of variation (CV) was 2.1%, and the corresponding interassay CV was 2.2% (Sigma Diagnostics, Deisenhofen, Germany). The reference interval for fasting values was 20 to 410 μmol/L in our laboratory.

HbF was measured with the high pressure liquid chromatography (HPLC) method according to Jeppsson et al.[10] The chromatographic system consisted of a 4000 autosampler, an L-6200 intelligent pump, an L-5025 column heater, and an L-4250 UV-VIS detector (all from E. Merck, Darmstadt, Germany). System controlling and calculations were performed with the D-7000-HPLC-Manager software (Merck). Kation-exchange chromatography was performed with a Mono-S-HR 5/5 column (Kabi Pharmacia Diagnostics, Uppsala, Sweden). The HbF intra-assay CV was 0.1%, and the corresponding interassay CV was 1.8% at an HbF level of 2.1%. Because HbF continues to decrease throughout adult life[9] and study subjects were in part adolescents, the reference interval for adults was not valid for our study population. Therefore, we defined an elevated HbF fraction as a value above the 95% percentile of the control group (HbF >0.87%). $HbA1_c$ was also measured with this HPLC method (normal range, 4.2% to 6.8%).

Other reference intervals for our laboratory were essentially equal to those listed by Laposata.[11]

Statistical analysis. The results are means ± SEM. A sample size of 30 and 40 subjects in the experimental and control group, respectively, can detect a difference of 20% in proportion of subjects with elevated HbF with a power of 0.80 ($\alpha = 0.05$). To test differences of variables between the groups, the Mann-Whitney U test was applied. Differences between the number of subjects with an elevated HbF were analyzed with the χ^2 test.

For coping with confounder variables, we applied two strategies: (1) matching in the design phase and (2) statistical adjustment in the analysis phase. Because more than one variable was found different in the two groups, we used a multivariate analysis for adjustment. Constructing and validating a multivariate model of the effects of covariates on outcome represents an attempt to remove much or most of the effects of covariates from the observed results.[12] To avoid possible

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Table 1. Clinical Characteristics of Study Patients With Anorexia or Bulimia and Healthy Controls

Clinical Characteristics	Control Subjects (n = 42)	Anorexia/ Bulimia Patients (n = 31)
Age (yr)	24.8 ± 0.85	25.0 ± 0.87
Body mass index (kg/m²)	22.5 ± 0.6	20.9 ± 1.2*
$HbA1_c$ (%)	5.5 ± 0.1	5.5 ± 0.1
Hb (g/L)	136 ± 1	138 ± 2
Hematocrit	0.39 ± 0.00	0.42 ± 0.01†
Erythrocyte count (10^{12}/L)	4.4 ± 0.0	4.7 ± 0.1†
Mean corpuscular volume (fL)	88.6 ± 0.5	89.5 ± 0.9
Reticulocyte count (10^{12}/L)	85.5 ± 5.0	88.1 ± 6.4
Leukocyte count (10^9/L)	7.9 ± 0.3	5.9 ± 0.3‡
Platelet count (10^9/L)	240 ± 10	260 ± 10
Iron (μmol/L)	18 ± 1	21 ± 2
Ferritin (μg/L)	40 ± 0	50 ± 1
Transferrin (g/L)	3.51 ± 0.07	3.06 ± 0.09‡
Bilirubin (μmol/L)	8 ± 1	8 ± 1
Aspartate aminotransferase (U/L)	9 ± 1	10 ± 1
Lactate dehydrogenase (U/L)	154 ± 4	147 ± 6
Haptoglobin (g/L)	1.01 ± 0.05	1.09 ± 0.08
Potassium (mmol/L)	4.4 ± 0.7	4.1 ± 0.9

Values are expressed as means ± SEM.
*$P < .05$.
†$P < .005$.
‡$P < .0005$.

violations of distribution assumptions (normality, homoscedasticity),[13] we did not apply linear but rather applied dichotomous logistic regression analysis to our data.[14] Because HbF continues to decrease throughout adult life, age was included in the logistic regression model irrespective of statistical significance. The variable β-hydroxybutyrate and additional covariates (hematologic and metabolic abnormalities) were conditionally selected in a forward stepwise procedure (inclusion criteria, $P < .05$). Calculations were performed using the SPSS statistics program Version 6.0 (SPSS Inc, Chicago, IL).

RESULTS

The clinical characteristics of the two groups were not different as far as age, sex, $HbA1_c$, Hb, mean corpuscular volume, reticulocyte count, platelet count, iron, ferritin, bilirubin, aspartate aminotransferase, lactate dehydrogenase, haptoglobin, and potassium were concerned (Table 1). However, β-hydroxybutyrate levels were higher in the patients than in the controls (460 ± 90 *v* 110 ± 20 μmol/L, $P < .0001$; Fig 1A). In addition, we observed distinct differences in the possible interfering variables body mass index, leukocyte count, transferrin, hematocrit, and erythrocyte count (Table 1). Subjects with an elevated HbF fraction (>0.87%) were observed four times as often in patients than in controls (29 *v* 7%, $P = .0126$; Fig 1B).

HbF and β-hydroxybutyrate levels were associated, as shown in the original data plot (Fig 2). We applied multivariate logistic regression models to assess which variables might explain the differences in HbF between patients and controls (Tables 2 and 3). Logistic regression of elevated HbF (Table 2) showed a significant age-adjusted effect of β-hydroxybutyrate ($P = .005$). There was no significant effect of other possible interfering covariates: body mass index, Hb, hematocrit, erythrocytes, reticulocytes, leukocytes, platelets, iron, ferritin, transferrin, bilirubin, aspartate aminotransferase, lactate dehydrogenase, haptoglobin, potassium, and group (patients/controls). The latter variable "group (patients/controls)" summarizes the effects of variables that could have accounted for differences in HbF between patients and controls but have not explicitly been defined in the present study. The effect of this group-variable was nonsignificant, making a major effect of unidentified variables unlikely. To confirm the effect of β-hydroxybutyrate, we removed β-hydroxybutyrate from an alternate regression model (Table 3). In this model, only the effect of the group-variable (patients/controls) became significant ($P = .02$). Thus, from among the variables investigated in this study, β-hydroxybutyrate exclusively explained the differences in HbF elevations between patients and controls.

DISCUSSION

Our data show that a modest level of ketonemia, generated from unrestrained lipolysis, is related to an increased synthesis of HbF. Specifically, we found a strong association between β-hydroxybutyrate and elevated HbF in young women with starvation ketosis. In several other experimental and clinical conditions, ketones have been shown to induce the HbF production. The discovery that butyrate is an inducer of HbF arose from the observation that the switch from HbF ($\alpha_2\gamma_2$) to

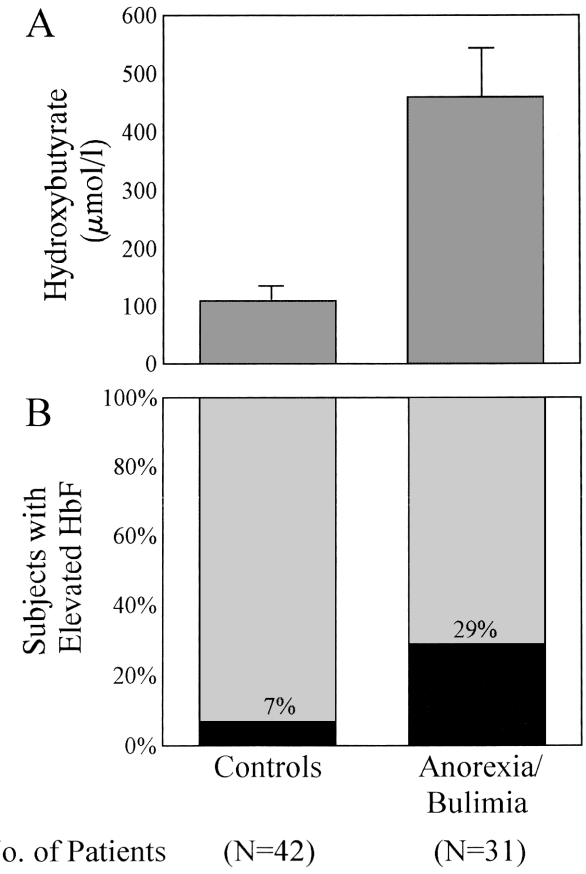

Fig 1. β-Hydroxybutyrate levels ($P < .0001$; A) and proportions of subjects with elevated HbF fraction (>0.87%) ($P = .0126$; B) in female control subjects and in women suffering from anorexia nervosa or bulimia.

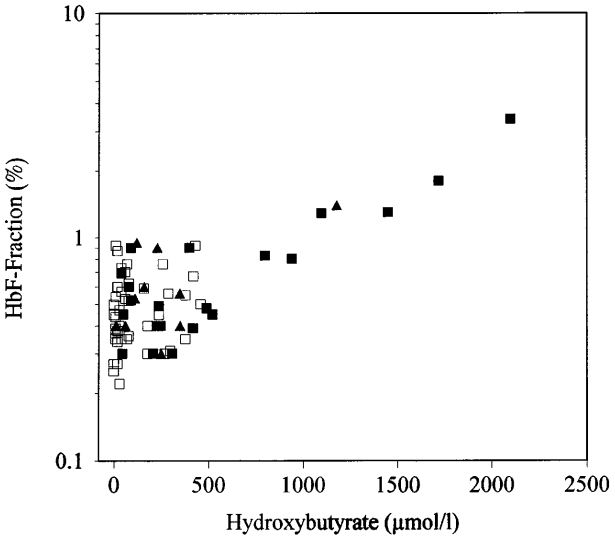

Fig 2. Scatterplot of HbF and β-hydroxybutyrate levels in female control subjects (□) and in women suffering from anorexia nervosa (▲) or bulimia (■) .

Hb A ($\alpha_2\beta_2$) is delayed in infants of diabetic mothers. In these newborns, elevated plasma concentrations of a labile analogue of butyric acid, α-amino-*n*-butyric acid, were reported.[15] Second, in vivo and in vitro experiments have confirmed that butyrates are potent stimuli of γ-globin mRNA and protein production.[16,17] Third, a clinical pilot trial in patients with β-hemoglobinopathies showed that butyrate can increase fetal-globin production within weeks to levels that can ameliorate β-globin disorders.[1] Finally, a case has been reported with an induction of HbF in the presence of increased 3-hydroxybutyric acid associated with β-ketothiolase deficiency.[18] This natural in vivo experiment indicated that the induction of HbF requires a sustained presence of high butyrate concentrations. In addition to these various conditions, our findings clearly showed that in starvation ketosis β-hydroxybutyrate was generated in sufficient large concentrations to stimulate a clinically measurable HbF production.

The mechanism by which butyrate achieves HbF induction is unknown. Butyrate stimulates a specific embryonic ρ-globin gene in adult chickens through 5′ flanking sequences[19] and selectively stimulates the γ-gene in fetal sheep, cultured human erythroid cells, and adult nonhuman primates.[16,17,20] Butyrate may act through sequences near the transcriptional start site to stimulate the activity of the human γ-globin gene promoter.[21] Because butyrate is known to inhibit the enzyme histone deacetylase[22] and to increase the acetylation of histones in other systems, Perrine et al postulated that this activity could be involved in the action of butyrate on γ-globin expression.[21] In the biochemical pathway of butyrate catabolism, butyrate enters mitochondria, where it undergoes β-oxidation to form acetate in a molar ratio of 2:1. Both butyrate and acetate, as well as chemical derivates of these compounds, can induce HbF.[23,24] We do not know yet whether each of these compounds acts directly to induce HbF or whether they are converted to one or more metabolic intermediates that subsequently exert their effect. Like many conditions resulting in increased HbF in adult life, ketonemia appears to involve an increased erythropoietic drive that results in a higher proportion of erythroid progenitor cells activating their inherent ability to synthesize low amounts of HbF.

Table 2. Logistic Regression of Elevated HbF Level on β-Hydroxybutyrate and Covariates

Independent Variables	Regression Coefficient	SE	*P* Value
Age (yr)	0.0478	0.0661	.4693
β-Hydroxybutyrate (µmol/L)	0.0024	0.0009	.0050

Age was included in the model irrespective of statistical significance. The variable β-hydroxybutyrate and additional covariates were conditionally selected in a forward stepwise procedure (inclusion criteria $P < .05$). The nonsignificant variables that were not included in this model using this selection method were body mass index, Hb, hematocrit, erythrocyte, reticulocyte, leukocyte, platelet count, iron, ferritin, transferrin, bilirubin, aspartate aminotransferase, lactate dehydrogenase, haptoglobin, potassium, and group (patients/controls).

Table 3. Logistic Regression of Elevated HbF Level on Covariates (Without β-Hydroxybutyrate)

Independent Variables	Regression Coefficient	SE	*P* Value
Age (yr)	0.0368	0.0652	.5725
Group (patients/controls)	1.6752	0.7199	.0200

In this model, β-hydroxybutyrate has been omitted from the list of variables for the conditional forward selection. Again, age was included in the model irrespective of statistical significance. The nonsignificant variables that were not included in this model using this selection method were body mass index, Hb, hematocrit, erythrocyte, reticulocyte, leukocyte, platelet count, iron, ferritin, transferrin, bilirubin, aspartate aminotransferase, lactate dehydrogenase, haptoglobin, and potassium.

In addition to ketones, we have considered other possible interfering variables in our calculations that are known to occur in eating disorders, ie, hematologic and metabolic abnormalities. Hematologic abnormalities are well recognized in anorexia, and several classes of pancytopenia due to bone marrow hypoplasia and, more rarely, to marrow cell necrosis have been reported.[25,26] These changes have been shown to be reversible after body weight was restored. In fact, we found lower leukocyte counts in the anorexia and bulimia patients. However, no relationship could otherwise be established in our data between blood cell counts, total Hb, the parameters of the iron metabolism, hemolysis parameters, and HbF, making an interfering effect of hematologic abnormalities unlikely. Metabolic abnormalities included weight loss, hypokalemia, and elevated liver enzymes.[27] Similarly, no relationship could have been established between body mass index, aspartate aminotransferase, serum potassium, and HbF. Thus, we consider it to be improbable that confounding effects, especially of hematologic and metabolic abnormalities, could have been the basis of the observed relationship between β-hydroxybutyrate and HbF.

Our findings are consistent with those of previous studies on type 1 diabetes and pregnancy. In type 1 diabetes, ketosis may occur as a result of poor metabolic control. It is often caused by insufficient insulin intake but may result from physical (eg, infection) or emotional stress, despite continued insulin therapy.

As a result of hypoinsulinemia, lipolysis is augmented, and acetoacetic and β-hydroxybutyric acids are produced. Our results suggest intermittent ketosis as a factor that could account for HbF elevations, as seen in type 1 diabetes.[4,28] In pregnancy, ketosis tends to develop in the presence of ''accelerated starvation.''[29] The hormonal and substrate milieu after an overnight 12-hour fast in pregnancy is comparable to that observed after a 36-hour fast in the nongravid state, hence the term ''accelerated starvation.'' Similarly, our results suggest ketosis as a factor that could account for maternal HbF production as seen in pregnancies.[5] To define the possible causes of HbF elevations in pregnancy is relevant because of the differential diagnosis of transplacental hemorrhage[30] and because gestational ketonemia imposes a risk on fetal development.[31]

In conclusion, we have been able to show that β-hydroxybutyrate generated in starvation is associated with increased levels of HbF. Thus, unrestrained lipolysis can produce β-hydroxybutyrate in sufficient quantities to induce a clinically measurable amount of HbF. Our findings suggest that, also in type 1 diabetes and pregnancy, intermittent ketosis might stimulate HbF production.

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