Exogenous D- β -Hydroxybutyrate Lowers Blood Glucose by Decreasing the Availability of L-Alanine for Gluconeogenesis

Adrian Soto Mota (✉ adrian.soto@dpag.ox.ac.uk)

University of Oxford

Nicholas Norwitz

University of Oxford

Rhys Evans

University of Oxford

Kieran Clarke

University of Oxford

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Abstract

Background

Interventions that acutely increase blood ketone concentrations simultaneously lower blood glucose levels, although the explanation for this phenomenon is unknown. The hypoglycaemic effect of acute ketosis is greater in people with type 2 diabetes (T2D) in whom gluconeogenesis contributes significantly to their hyperglycaemia. One gluconeogenic substrate secreted by skeletal muscle at higher levels in people with T2D is L-alanine. Infusion of ketones lowers circulating L-alanine blood levels, so here we sought to determine whether supplementation with L-alanine would attenuate the hypoglycaemic effect of a ketone ester (KE) drink.

Methods

This crossover study involved two separate visits for 10 healthy human volunteers who fasted for 24 hours prior to the ingestion of 25 g of d- β -hydroxybutyrate (β HB) monoester (ΔG°). During one of the visits participants ingested 2 g of L-alanine. Blood L-alanine, L-glutamine, glucose, β HB, free fatty acids (FFA), lactate, and C-peptide were measured.

Findings

The KE drinks elevated blood β HB concentrations from negligible to 4.5 ± 1.24 mM/l, and lowered both glucose, from 4.97 to 3.77 ± 0.4 mM/l, and L-alanine from 0.56 to 0.41 ± 0.9 mM/l. L-alanine in the KE drink elevated blood L-Alanine to $0.56 \pm$ mM/l, but had no significant effect on blood β HB, L-glutamine, FFA, lactate, or C-peptide concentrations. Preventing the KE-induced decrease in L-alanine by supplementing it significantly attenuated the glucose drop from mean 28% to 16% ($p < 0.001$). (Illustrated in Figure 1).

Conclusions

The hypoglycaemic effect of acutely elevated β HB is, at least partially, due to β HB decreasing L-alanine availability as a substrate for gluconeogenesis.

Introduction

In the fed state, the human brain relies almost exclusively on glucose as an energy source. However, dietary carbohydrates are not always available, so glucose can be derived from through gluconeogenesis. But gluconeogenesis alone is not compatible with human life as it requires the catabolism of lean muscle tissue and, thus, even relatively short periods without carbohydrate would lead to severe muscle wasting and death. Ketone bodies, primarily d- β -hydroxybutyrate (β HB), are derived from fat stores to provide an alternative fuel substrate for the brain. Not only does β HB supplant glucose as a brain fuel substrate, but it inhibits gluconeogenesis and, thus, spares lean muscle tissue¹.

In type 2 diabetes (T2D), excessive gluconeogenesis underlies hyperglycemia². Because β HB inhibits gluconeogenesis³, it may be useful in managing hyperglycemia and ketogenic diets successfully reverse T2D^{4,5}. Carbohydrate restriction certainly plays a role in glycaemic control, it is also true that, in both animals and humans, exogenous infusion of β HB decreases blood glucose⁶⁻⁹. Therefore, the development of exogenous ketones for human consumption has inspired interest in exogenous ketosis as a potential treatment for this T2D¹⁰, especially because the hypoglycemic effect of ketosis is larger in people living with T2D than in healthy controls¹¹. However, no mechanism describing this difference has been elucidated.

Gluconeogenesis is main hyperglycaemic mechanism in people living with T2D^{2,12}. Ketosis preserves gluconeogenic muscle tissue¹³ and ketones limit the secretion of L-alanine, a gluconeogenic substrate, by muscle cells^{11,14}. Thus, it is plausible that ketones decrease blood glucose (and do so to a greater extent in those with T2D) by reducing L-alanine availability for gluconeogenesis. This study sought to test this hypothesis by investigating whether L-alanine supplementation attenuates hypoglycaemic effect of exogenously induced ketosis.

Methods

I) Ethics

This study was pre-registered at ISRCTN16169021 and received Ethics approval by the East of England - Cambridge South Research Ethics Committee on February 18th, 2019 (Reference 18/EE/0115). All participants were older than 18 years old and signed an informed consent form approved by the same Research Ethics Committee.

This study was conducted in accordance with the guidelines set forth by the International Conference on Harmonisation Guidelines for Good Clinical Practice, and the Declaration of Helsinki regarding the treatment of human subjects in a study³².

II) Statistical analysis

Sample size was calculated to detect a blood glucose difference of at least 0.9 mM between groups with a statistical power of 80% and an alpha probability of 0.05. Therefore, six participants per group were needed. To achieve a sex and age balanced cohort and to allow for dropouts, a total of 10 participants were recruited and all completed the study. Sample size calculations were performed using G*Power version 3.1³¹.

All statistical tests and analysis were performed using Excel[®] (Microsoft, United States). Data, presented as means $\pm$ standard deviations (SD), were analysed using One-way repeated measurements ANOVA and adjusted with Bonferroni's correction for multiple comparisons. Differences were considered significant at $p < 0.05$. Since baseline blood concentrations of L-Alanine and L-Glutamine have large interpersonal variations due to body composition, comparisons were made using change from baseline.

III) Materials and procedures

Participants fasted for 24 hours. At each visit, participants drank a mix 25 ml of the $\Delta G^{\text{®}}$ ketone monoester, (R)-3-hydroxy butyl (R)-3-hydroxybutyrate (TdeltaS Ltd., United Kingdom), diluted in 25 ml water.

After ingestion, the β HB monoester bond is cleaved by esterases in the gut wall, yielding β HB and butanediol in equal amounts. Both are absorbed into the portal circulation and the latter is taken up by the liver, where it is converted into β HB by alcohol dehydrogenase (ADH). β HB leaves hepatocytes via monocarboxylate transporters. Pharmacokinetic studies have shown that, in the fasted and resting states, the β HB monoester can induce ketoses for 3–4 h, with a peak at ~ 1 h that is dose-dependent³³.

On their second visit, to match skeletal muscle release of L-alanine per two hours², participants drank 2g of L-alanine (Hard Eight Nutrition LLC, United States) in water.

Blood samples were collected through a venous cannula at baseline and every 15 minutes for two hours. Glucose, β HB, lactate and free fatty acids were assayed using a commercial semi-automated bench-top analyser (ABX Pentra,

Montpellier, France). L-alanine, L-glutamine and human C-peptide were measured using kits (by Abcam, United Kingdom: ab83394, ab145659 and ab178641 respectively).

Results

Participants' anthropometric characteristics

Five healthy males and five healthy females aged 40 years old (+/- 16) were enrolled. Ingestion of the ΔG® ketone monoester drink was well-tolerated by all participants and none presented symptomatic hypoglycaemia. All participants were healthy, not overweight, and included five women and five men. Their mean age was 40 years old (SD = 16 years) and their mean BMI: 23.2 kg/m2 (SD = 2.4). Table 1 summarises anthropometric characteristics of our participants.

Table 1. Participant characteristics

Sex	5 (F), 5 (M)
Age (years)	40 (16)
Height (cm)	176 (15)
Weight (kg)	73 (18)
BMI (kg/m ²)	23.2 (2.4)

Data are expressed as mean (Standard deviation).

Blood L-alanine, L-glutamine and glucose concentration changes after inducing acute ketosis without and with L-alanine supplementation.

In the absence of L-alanine supplementation, inducing acute ketosis with the ΔG® ketone monoester raised blood βHB concentration to 4.5 mM/l ± 1.24mM/l and L-glutamine by 9% ± 4% (absolute change from 1.099 to 1.223 mM/l ± 0.045 mM/l). By contrast, exogenous ketosis lowered blood L-alanine by 26% ± 9.3% (absolute change from 0.563 to 0.4409 mM/l ± 0.053 mM/l) and blood glucose by 28% ± 8% (absolute change from 4.97 to 3.77 mM/l ± 0.4 mM/l). Compared to baseline, changes in βHB, glucose, and alanine, but not glutamine, were significantly different (p < 0.05).

In the presence of L-alanine supplementation, inducing ketosis raised blood βHB concentration to 4.6 ± 1.35 mM/l and L-glutamine by 10% ± 6% (absolute change from 1.12 to 1.26 ± 0.05 mM/l). As expected, L-alanine supplementation led to a net increase in L-alanine by 16% (absolute change from 0.543 to 0.682 μmol/L ± 0.051 mM/l), thus maintaining L-alanine availability for gluconeogenesis. Correspondingly, blood glucose decreased by a lesser amount, only by 17% ± 5% (absolute change from 5.01 to 4.03 mM/l ± 0.3 mM/l). Again, changes in βHB, glucose and L-alanine, but not L-glutamine, were significant (p<0.05). These results are described in detail in Table 2 and illustrated in Figure 2.

Table 2. Blood L-alanine, L-glutamine and glucose concentration changes after inducing acute ketosis with and without L-alanine supplementation

a) Absolute changes	Timepoint	β HB (mM/l)	β HB + A (mM/l)	Glucose (mM/l)	Glucose + A (mM/l)	Glutamine (mM/l)	Glutamine + A (mM/l)	Alanine (mM/l)	Alanine + A (mM/l)
	0	0.6	0.5	4.97	5.01	1.099	1.120	0.563	0.543
	15	3.4	3.2	4.52	4.64	1.120	1.130	0.492	0.579
	30	3.9	4.0	4.11	4.31	1.143	1.170	0.432	0.635
	45	3.8	4.3	3.77	4.03	1.187	1.210	0.409	0.682
	60	4.3	4.7	3.79	4.07	1.223	1.260	0.417	0.641
	75	4.5	4.6	3.91	4.15	1.211	1.190	0.444	0.599
	90	4.1	4.2	3.99	4.37	1.190	1.210	0.492	0.557
	120	3.7	3.6	4.1	4.51	1.130	1.120	0.515	0.541
b) Relative changes from BL (%)	Timepoint	β HB	β HB	Glucose	Glucose + A	Glutamine	Glutamine + A	Alanine	Alanine + A
	0	0	0	0	0	0	0	0	0
	15	566.7	627.5	-9	-4	7	5	-26	10
	30	650.0	778.4	-18	-13	9	10	-24	13
	45	633.3	843.1	-28	-17	7	9	-25	11
	60	716.7	921.6	-24	-16	6	4	-25	16
	75	750.0	892.2	-24	-12	4	1	-24	12
	90	683.3	825.5	-22	-10	0	5	-13	8
	120	616.7	705.9	-19	-9	4	10	-11	5

Data are expressed as means from n=10.

All measurements were performed by triplicate.

L-alanine supplementation attenuated the hypoglycaemic effect of acute ketosis.

The magnitude of the hypoglycaemic effect of exogenous ketosis was significantly smaller when L-alanine was supplemented, as compared to when it was not, at 45 and 60 min after $\Delta G^{\text{®}}$ administration ($p < 0.001$). Glucose levels were consistently higher, relative to baseline, at all timepoints when L-alanine was supplemented as compared to when it was not (Figure 3).

L-Alanine supplementation did not impact changes in blood lactate, free fatty acids (FFA) and C-peptide during acute ketosis.

Blood lactate, FFA and C-peptide concentrations were similar at all timepoints after inducing ketosis with $\Delta G^{\text{®}}$ regardless of L-alanine supplementation (illustrated in Figure 4).

Discussion

These data demonstrate that a decrease in L-alanine availability contributes to the hypoglycaemic effect of acute ketosis. The magnitude of the effect suggests that β HB-mediated decrease in L-alanine levels is a major contributor to this phenomenon; however, it is also evident that the hypoglycaemic effect of ketosis is a result of multiple mechanisms.

Pyruvate in disguise

During prolonged fasting, L-alanine blood levels decrease more so than those of any other amino acid, largely because of hepatic uptake of L-alanine to fuel gluconeogenesis. This phenomenon prompted the discovery of the glucose-alanine cycle, in which pyruvate is transformed into L-alanine via transamination in skeletal muscle. L-alanine (pyruvate in disguise) is released to the bloodstream, taken up by the liver, and transformed back into pyruvate to fuel gluconeogenesis¹⁵. Thus, any reduction in intramuscular pyruvate would result in lower L-alanine levels in the blood and less gluconeogenesis.

Ketone oxidation decreases intramuscular pyruvate via two mechanisms. First, ketone oxidation reduces glycolysis in skeletal muscles¹⁴ and, therefore, decreases pyruvate production. Second, ketone body oxidation increases Acetyl CoA levels¹⁶. Acetyl CoA is an allosteric activator of pyruvate carboxylase, thus, driving the conversion of pyruvate to oxaloacetate¹⁷. In addition to decreasing pyruvate for transamination into L-alanine, ketosis also directly decreases L-alanine production by reducing skeletal muscle protein degradation¹³.

Furthermore, in the liver, L-alanine supports gluconeogenesis. L-alanine allosterically inhibits the liver isozyme of pyruvate kinase, the enzyme responsible for the last step of glycolysis¹⁸. Thus, L-alanine restriction would favour glycolysis over gluconeogenesis. L-alanine is also a potent glucagon secretion agonist¹⁹, and glucagon stimulates gluconeogenesis. Again, L-alanine restriction is predicted to downregulate gluconeogenesis in the liver.

These links between ketone metabolism and gluconeogenesis resonate with the evolutionary function of ketosis: to spare gluconeogenic muscle tissue.

The hypoglycaemic effect of ketone body oxidation is likely pleiotropic.

Exogenously induced ketosis can stimulate insulin secretion^{20,21}; however, the insulin change is not large enough to account for the entire hypoglycaemic effect. There is also evidence that it is not an increase in glucose clearance from the blood, but rather a decrease in glucose secretion into the blood that accounts for most of the hypoglycaemic effect of ketosis³. Furthermore, ketone salt infusions in patients with type 1 diabetes who were off insulin demonstrate that acute ketosis still lowers blood glucose, even in the absence of insulin¹¹.

The hypoglycaemic effect is not restricted to the fasting state. Inducing acute ketosis lowers the postprandial glycaemic curve after a dextrose challenge without inducing significant differences in insulin secretion²². One of the proposed mechanisms is that β HB-mediated inhibition of lipolysis²³ depletes the blood supply of fatty acids, driving an increase in glucose uptake. However, this has not been observed after the ingestion of niacin, which also blocks lipolysis and has no effect on gluconeogenesis²⁴. Moreover, while inhibiting lipolysis would restrict the amount of glycerol available to fuel gluconeogenesis, in the fasting human, L-alanine and lactate, not glycerol, are the main relative contributors to liver gluconeogenesis²⁵. Figure 5 summarises the mechanisms whereby exogenously induced acute ketosis lowers blood glucose concentration.

Limitations of this study

First, the relative of L-alanine to gluconeogenesis varies depending on whether a person is in the fed or fasted state^{26,27}. Participants in this study were all fasted, and this was necessary for control purposes. Future studies may choose to investigate the same question in postprandial participants. Second, the contribution of a ketone ester liver-specific effect cannot be ruled out.

However, the liver cannot oxidise ketone bodies²⁸ and, while the hepatic conversion of butanediol derived from ketone ester into β HB would alter hepatocyte NAD/NADH balance (which could impact endogenous glucose production), studies comparing the ingestion of equimolar quantities of ketone salts and ketone esters (that lack a butanediol component) demonstrate hypoglycaemic effects of similar magnitudes⁹.

Future Directions

These data demonstrate, in healthy humans, that a reduction in L-alanine availability to fuel gluconeogenesis is a major contributor to the hypoglycaemic effects of acute ketosis. Because gluconeogenesis is pathologically elevated during insulin resistance, and gluconeogenesis is a major contributor to poor glycaemic control in T2D, future studies will investigate the potential for exogenous ketosis to manage hyperglycaemia in T2D.

Declarations

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AUTHOR CONTRIBUTIONS

All authors contributed significantly to the design, execution, analysis and reporting of this study.

DECLARATION OF INTERESTS

The intellectual property covering the uses of ketone bodies and ketone esters are owned by BTG Plc, Oxford University Innovation Ltd and the US National Institutes of Health. Professor Kieran Clarke, as an inventor, will receive a share of the royalties under the terms prescribed by each institution. Professor Clarke is a director of TdeltaS Ltd, a company spun out of the University of Oxford to develop products based on the science of ketone bodies in human nutrition. The other authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Figures

Figure 1

Blood L-alanine, L-glutamine and glucose concentration changes after inducing acute ketosis with and without L-alanine supplementation Data are presented as the mean $\pm$ SD (n = 10 participants, measurements by triplicate).

Figure 2

L-alanine supplementation attenuated the hypoglycaemic effect of acute ketosis. Data are presented as mean AUC (n = 10 participants, measurements by triplicate). Y axis = percent change from baseline of blood glucose concentration (mM). X axis = Time (minutes) after drinking 25 ml (containing 25 g) of β HB monoester. Mean change after baseline at every timepoint was analysed with a one-way repeated measures ANOVA and found to be statistically significant after a Bonferroni correction for multiple comparisons ($p < 0.006$).

Figure 3

Blood lactate, free fatty acids (FFA) and C-peptide during acute ketosis with and without L-alanine. Mean change after baseline was analysed with a one-way repeated measures ANOVA. Statistical significance after a Bonferroni correction ($p < 0.001$).

Figure 4

Summary of the mechanisms whereby ketosis lowers blood glucose In skeletal muscle: β HB increases the concentration of acetyl CoA¹⁶, which inhibits pyruvate dehydrogenase and activates pyruvate carboxylase¹⁷. As a result, more pyruvate is transformed into oxaloacetate. Additionally, the rise in acetyl CoA inhibits phosphofructokinase-1 (PFK1), downregulating glycolysis, and therefore, decreasing pyruvate production²⁹. Through these two mechanisms, pyruvate levels are reduced and there is less pyruvate available for transamination into L-alanine. β HB also decreases protein breakdown, further reducing L-alanine production¹⁴. In adipose tissue: β HB inhibits lipolysis via de PUMA-G receptor²³, reducing the release of glycerol, a minor gluconeogenic substrate. In the pancreas: β HB promotes insulin release by the beta cells^{21,30}. Furthermore, there is less L-alanine to stimulate glucagon release by the alpha cells¹⁹. The decrease in gluconeogenic substrates (L-alanine and glycerol) and inhibitory hormonal signaling (increase in insulin/glucagon ratio; and L-alanine allosterically inhibits pyruvate kinase¹⁸) cause a decrease in gluconeogenesis by the liver. The net result of acute ketosis induced by exogenous β HB is a small increase in peripheral glucose uptake, due to a small increase in insulin, and a larger decrease in gluconeogenesis and glucose release by the liver.

Supplementary Files

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- [Fig01.png](#)