The influence of calcium supplementation on substrate metabolism during exercise in humans: a randomized controlled trial.

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Abstract

**Background/Objectives:** High calcium intakes enhance fat loss under restricted energy intake. Mechanisms explaining this may involve reduced dietary fat absorption, enhanced lipid utilization and (or) reductions in appetite. This study aimed to assess the impact of two weeks of calcium supplementation on substrate utilization during exercise and appetite sensations at rest. **Subjects/Methods:** Thirteen physically-active males completed two 14-d supplemental periods, in a double-blind, randomized crossover design separated by a ≥ 4 week washout period. During supplementation, a test-drink was consumed daily containing 400 and 1400 mg of calcium during control (CON) and high-calcium (CAL) periods, respectively. Cycling-based exercise tests were conducted before and after each supplemental period to determine substrate utilization rates and circulating metabolic markers (non-esterified fatty acid, glycerol, glucose and lactate concentrations) across a range of exercise intensities. Visual analogue scales were completed fasted and at rest, to determine subjective appetite sensations. **Results:** No significant differences between supplements were observed in lipid or carbohydrate utilization rates, nor in circulating metabolic markers (both \( P > 0.05 \)). Maximum rates of lipid utilization were 0.47 ± 0.05 and 0.44 ± 0.05 g/min, for CON and CAL respectively, prior to supplementation, and 0.44 ± 0.05 g/min respectively, post-
supplementation (main effects of time, supplement, and time x supplement interaction effect all \( P > 0.05 \)). Furthermore, no significant differences were detected in any subjective appetite sensations (all \( P > 0.05 \)). **Conclusions:** Two weeks of calcium supplementation does not influence substrate utilization during exercise in physically-active males.

**Key words:** appetite, dairy, fat oxidation, lipid utilization, non-esterified fatty acids.
Introduction

A growing body of research implicates calcium intake in adipose regulation. Epidemiology links high-calcium intake with reduced body fat percentage\textsuperscript{1}, and interventions demonstrate accelerated loss of adipose tissue\textsuperscript{2}. Alongside decreased dietary fat absorption\textsuperscript{3}, mechanisms possibly contributing to this effect are, elevated lipid utilization\textsuperscript{4, 5} and/or reduced appetite\textsuperscript{6-8}. Consumption of high-calcium meals have been shown to enhance postprandial lipid utilization by some\textsuperscript{8, 9} but not others\textsuperscript{6, 7, 10}. Meta-analyses suggest that evidence for chronic (>7 d) calcium intake (increase of ~958 mg/d) and lipid utilization, is stronger than acute effects\textsuperscript{4}. The reduction in appetite observed with acute calcium intake\textsuperscript{6-8}, may translate into a chronic reduction in appetite\textsuperscript{11-14}.

The mechanism(s) underlying a change in fat utilization with calcium intake, in humans, is not clear. Most evidence comes from \textit{in vitro} or animal data, and the most prominent theory is currently that of parathyroid hormone (PTH) and/or 1,25(OH)\textsubscript{2}D regulating lipid metabolism\textsuperscript{15, 16}. Dietary manipulation of 1,25(OH)\textsubscript{2}D in humans (oral vitamin D combined with low-calcium diet) however, does not influence whole-body lipid oxidation or lipolytic markers\textsuperscript{17}. Similarly, increased lipid oxidation with calcium supplementation has been observed in the presence of similar PTH\textsuperscript{18}, suggesting other mechanisms may be at play.
Increasing the calcium content of a meal by ~900 mg potentiates postprandial circulating glucose-dependent insulinotropic peptide\textsubscript{1-42} (GIP\textsubscript{1-42}), glucagon-like peptide-1 (GLP-1) and insulin concentrations\textsuperscript{7}. GLP-1 and insulin suppress appetite\textsuperscript{19-21} and emerging evidence suggests that GIP and GLP-1 influence lipid metabolism. GIP stimulates adipose fatty acid re-esterification under hyperinsulinemic hyperglycemia\textsuperscript{22}. GLP-1 positively associates with fasting lipid utilization\textsuperscript{23}, and dipeptidyl peptidase-IV (DPP-IV; an enzyme that degrades the receptor-active form of GIP and GLP-1, but also of other peptides) inhibition, increases lipid utilization\textsuperscript{24}.

The effect size that an elevated calcium intake has on lipid oxidation, tends to be greater when under energy deficit\textsuperscript{4}. Melanson \textit{et al.} used diet and exercise to produce negative energy balance after 7 d of high-calcium intake\textsuperscript{18}. The respiratory quotient was only significantly lower during low-intensity (bench stepping) exercise with high-calcium intake. To our knowledge, this is the only study to examine the effect of a week of elevated calcium intake on lipid oxidation during exercise, and as bench stepping was standardized, it was not possible to discern the effect on maximal lipid utilization.

We therefore hypothesized that repeated (daily for 14 d) elevated exposure of GIP\textsubscript{1-42} and GLP-1 from calcium ingestion (as seen with a similar dose acutely\textsuperscript{7}) may influence lipid utilization during exercise. Accordingly, we aimed to
investigate the effect of 2 weeks of high-calcium intake (increase of ~1000 mg) on maximal lipid utilization during exercise. We also aimed to determine the impact of calcium supplementation on fasting concentrations of plasma GIP, GLP-1 and insulin, along with subjective appetite sensations.

**Methods**

**Participants**

The sample size was based on the effect size (11%) for lipid utilization obtained by meta-analyses and the typical error of the maximum rate of lipid utilization during exercise of 9%. This calculated that 13 participants should provide statistical power above 80% with an alpha level of 0.05.

Thirteen physically active males were recruited from the student and staff population of Northumbria University between October 2012 and February 2013. Participants’ age, stature, body mass and habitual calcium intakes were (mean ± SD: 26 ± 4 y, 177 ± 6 cm, 78 ± 6 kg, 1040 ± 445 mg/d; for aerobic capacity see Table 2). Participants were self-reportedly physically active (moderate-vigorous activity, ≥30 min, 5 times/week), non-smokers, and free of metabolic disease.

This study received ethical approval from the Faculty of Health and Life Sciences Ethics Committee at Northumbria University, and participants provided written informed consent.
prior to the study. This study was registered at ClinicalTrials.gov (NCT01779245).

Study design

In a randomized (performed by J.T.G. using an online package: researchrandomizer.org), double-blind (both investigators and participants were blinded, an assistant who was not involved in data collection, prepared and labeled food packages), crossover design, participants underwent a control (CON) and a high-calcium (CAL) supplementation period. Before and after supplemental periods, exercise tests were conducted to determine substrate utilization (Figure 1). Prior to each exercise test participants were asked to replicate their diet for the preceding 24 h, and to abstain from exercise, caffeine and alcohol. Exercise tests began after a 10-14 h overnight fast, during which water was consumed *ad libitum*.

Exercise tests

Upon arrival at the laboratory, an intravenous catheter was inserted into an antecubital vein for repeated blood sampling. A 5-min resting sample of expired gas was collected in the supine position after an initial 5-min rest period.

Following baseline sampling, participants began the exercise test, established by others to assess maximal lipid utilization during exercise. Participants cycled on a
mechanically-braked cycle ergometer (Monark Ergomedic 874E, Vansbro, Sweden) at an initial power output of 95 W. Power output was increased by 35 W every 3 min until volitional tolerance, with cadence maintained at 70 revolutions/min. Expired gas samples were collected for the final min of each of the first 5 stages, and then for the final min of the test, when the participant indicated that they would only be able to continue for a further min. If the participant was unable to complete the full 60 s for the final sample, then the time was recorded. A minimum sample of 30 s was used for analysis. On occasions where the participant was able to continue past this “final” min, an additional sample was collected.

Sampling and analysis of expired gases
Expired gas analysis was performed using the Douglas bag technique (Supplementary Information 1) accounting for variance in ambient concentrations of oxygen and carbon dioxide.

Estimation of carbohydrate and lipid utilization
Substrate oxidation was estimated from oxygen consumption and carbon dioxide values using stoichiometric equations, adjusted for the contribution of glycogen to metabolism.
LO rate at rest and during exercise =
\( (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2) \)

CO rate at rest = \( (4.585 \times \dot{V}CO_2) - (3.226 \times \dot{V}O_2) \)

CO rate exercise at 40-50% \( \dot{V}O_2 \) peak = \( (4.344 \times \dot{V}CO_2) - (3.061 \times \dot{V}O_2) \)

CO rate exercise at 50-75% \( \dot{V}O_2 \) peak = \( (4.210 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2) \)

LO = lipid oxidation (g/min)

CO = carbohydrate oxidation (g/min)

\( \dot{V}O_2 \) and \( \dot{V}CO_2 \) are L/min

Carbohydrate utilization rates were only determined up until 75% \( \dot{V}O_2 \) peak29.

Supplemental protocol

Participants were provided with pre-packaged bags of milkshake powder to consume once daily. For both supplemental periods, bags contained cocoa powder (Tesco, Dundee, UK) and dried skimmed milk (Tesco, Dundee, UK) providing 987 kJ (235 kcal) energy; 13 g protein; 42 g carbohydrate and 1 g fat. For the CAL supplement, 4 g of a milk-extracted calcium powder (Capolac®, Arla Foods Ingredients amba, Denmark) was added to increase the calcium
content. Details of the supplementation, including independent verification are provided in Supplementary Information 2.

**Anthropometric variables**

Body mass was determined to the nearest 0.1 kg using balance scales (Seca, Birmingham, UK) upon arrival at the laboratory, where participants wore only light clothing. Stature was measured to the nearest 0.1 cm using a stadiometer (Seca, Birmingham, UK).

**Subjective ratings**

Subjective appetite ratings were assessed using previously validated, 100 mm VAS, upon arrival at the laboratory (in the fasted, resting state). Questions asked included: “how hungry do you feel?”, “how full do you feel?”, “how satisfied do you feel?” and “how much do you think you can eat?”. A combined-appetite score which has been used previously was also employed. Participants rated the palatability of the test drinks following consumption of the first drink using VAS with questions pertaining to visual appeal, smell, taste and overall palatability.

**Blood sampling and analysis**

Blood was sampled at baseline, during the final min of each of the first 5 stages, and immediately after termination of the
exercise test, to determine NEFA and glycerol (both kits from RX Daytona, Randox Laboratories Ltd, London, UK) and glucose and lactate (both measured by Biosen C_line, EKF Diagnostics, Magdeberg, Germany) concentrations. Samples were collected into EDTA Vacutainers and immediately centrifuged (10 min, 1509 g, 4°C). At baseline, an additional sample was collected into a non-anticoagulant tube and allowed to stand for 30 min before centrifugation (10 min, 1509 g, 4°C) for PTH (Immunotopics, San Clemente, USA) and insulin (IBL International GmbH, Hamburg, Germany) concentrations. A further EDTA tube with 25 μL aprotinin/mL whole blood was taken at baseline and immediately centrifuged (10 min, 1509 g, 4°C) for plasma GIP$_{1-42}$ (Immuno-Biological Laboratories Co., Ltd, Japan) and total GLP-1 (Epitope Diagnostics, San Diego, CA) concentrations. Samples were stored at -80°C before analysis. Inter-assay CVs for NEFA and glycerol were 1.2 and 2.1%, respectively. The intra-assay CV reported by the manufacturers was 4.74 and 1.34% for NEFA and glycerol, respectively.

Statistical analysis

Due to difficulties with blood sampling from one participant, data for all blood variables are $n = 12$ apart from when relationships between baseline variables were assessed. As all participants completed all trials, all other data are $n = 13$ and
are expressed as mean ± standard deviation (SD) unless stated otherwise. Data were tested for normal distribution using the Anderson-Darling normality test. Data not displaying normal distribution were log-transformed prior to statistical analysis. Insulin sensitivity was assessed by HOMA-IR$^{32}$. Two-way (supplement x time) repeated-measures ANOVA were used to detect differences between resting variables, maximal fat oxidation, total fat and carbohydrate oxidation, variables assessed at exhaustion and appetite. Three-way (supplement x time x power output) repeated-measures ANOVA were used to assess differences between circulating variables and substrate utilization during exercise. Following a significant interaction effect, Holm-Bonferroni post-hoc test was used to determine the location of variance and all $P$ values reported have been adjusted for multiple-comparisons. Differences between CON and CAL in the change from pre- to post-supplementation were assessed by paired t-tests. Differences were considered significant at $P < 0.05$. Relationships between variables were assessed by Pearson product-moment correlation coefficients.

Results

Palatability

Ratings of visual appeal (CON: 76 ± 14, CAL: 79 ± 14), smell (CON: 73 ± 15, CAL: 73 ± 17), taste (CON: 79 ± 13, CAL: 82
± 15) and palatability (CON: 78 ± 15, CAL: 82 ± 13) of the test drinks did not significantly differ (paired t-tests: $P = 0.467$, $P = 0.946$, $P = 0.464$, $P = 0.210$, respectively).

Resting measures

Resting energy expenditure, lipid and carbohydrate utilization, and circulating metabolites and hormones are displayed in Table 1. A significant interaction effect (supplement x time) was observed for insulin concentration and HOMA-IR, but not for any other resting variables (Table 1). Post-hoc analyses revealed that insulinemia was lower prior to supplementation with CAL vs. CON, but rose during supplementation to a concentration that was higher than both CON post-supplementation and CAL pre-supplementation (Table 1). The pre- to post-supplementation change in insulin concentrations was significantly different between CON and CAL as assessed by paired t-test ($P = 0.006$; Figure 2), as was HOMA-IR ($P = 0.029$).

No significant main (time or supplement), or interaction (supplement x time) effects were detected for any other resting variable (Table 1; all $P > 0.5$). Similarly, no differences were observed in body mass or subjective appetite (Supplementary Table 1).

Exercise measures
VO₂ peak did not significantly differ between tests (Table 2), nor did any of the metabolic variables measured (Table 2). A main effect of power output was detected for plasma lactate concentration and lipid and carbohydrate utilization (all \( P < 0.001 \)). However, no main effects for trial or interaction effects (supplement x time, supplement x power output, time x power, or supplement x time x power output) were observed for any exercise variable (all \( P > 0.05 \)). Figure 3 displays pre- to post-supplementation change in the maximum lipid utilization, which was not significantly different between CON and CAL (paired t-test, \( P = 0.619 \)). NEFA and glycerol concentrations during exercise are displayed in Figure 4, and glucose and lactate concentrations during exercise in Supplementary Figure 1.

Correlations between variables

No significant relationships were observed between the change in PTH and, resting, maximal or total lipid utilization (\( r = -0.09, r = 0.17 \) and \( r = 0.04 \), respectively, all \( P > 0.05 \)). Pre-supplementation (mean of CON and CAL) VO₂ peak however, inversely correlated with pre-supplementation insulinemia (Supplementary Figure 2; \( r = -0.65, P = 0.016 \)) and HOMA-IR \( (r = -0.72, P = 0.006) \).

Discussion
The main findings indicated by these data are three-fold. 1) two-weeks of calcium supplementation does not influence whole-body lipid metabolism during exercise, in young physically active males; 2) calcium supplementation does not influence circulating concentrations of GIP$_{1-42}$ or total GLP-1, or appetite sensations in the fasted state; 3) calcium supplementation elevated serum insulin concentrations in the fasted state.

High-calcium intake for 7 d increases 24-h lipid utilization in overweight individuals, achieved by increasing the consumption of dairy products$^{18}$. The change in substrate metabolism was most apparent during activity. Additionally, meta-analyses indicate that a ~1000 mg increase in calcium intake for ≥7 d increases fat utilization$^4$. Therefore, we investigated the effects of a ~1000 mg/d increase in calcium intake for 14 d, on fat utilization during exercise. In the present study, neither maximal fat utilization rates, nor the total amount of fat utilized during exercise were influenced by consumption of additional calcium. Other markers of substrate metabolism support this, as plasma NEFA, glycerol, glucose and lactate concentrations during exercise were unaffected by supplementation, suggesting lipolytic rate$^{33}$ and glycolytic flux$^{34}$ were unchanged.

Participants in the present study and that of Melanson et al.$^{18}$, were both non-obese, healthy and had similar habitual
calcium intakes (1040 mg/d vs. ~1100 mg/d, respectively). The only major difference in the participants was the use of physically active individuals in the present study. Consequently, the \( \dot{V}O_2 \) peak of the participants in the present study was \( \sim 51 \text{mL/kg/min} \), compared to \( \sim 32 \text{mL/kg/min} \). As training status has a major influence on substrate metabolism during exercise\(^{35,36}\), it is conceivable that calcium supplementation is only effective at increasing fat utilization in untrained individuals with limited capacity for fat oxidation. Notwithstanding this, given the higher basal insulin concentrations in response to calcium supplementation seen in the present study, it could be viewed that the absence of suppressed NEFA and glycerol concentrations, or rates of fat utilization somewhat corroborate the findings of Melanson et al.\(^{18}\) (greater fat utilization under similar insulinemia), as both indicate reduced insulin-suppression of lipid mobilization/utilization. A negative relationship between the change in PTH and the change in fat utilization in response to calcium supplementation was previously reported\(^{10}\), suggesting reduced PTH was (partially) responsible changes in fat oxidation seen by Gunther et al.\(^{37}\). PTH was unaffected by calcium supplementation in the present study, although others have shown enhanced fat oxidation with calcium supplementation with no change in PTH\(^{18}\). Hence it is presently unclear whether
PTH suppression is necessary for calcium supplementation to influence fat oxidation. Both the previous studies used dairy products to increase calcium intake, with potential for confounding from dairy protein, or other bioactive components\textsuperscript{38,39}.

The present study also indicates that calcium supplementation does not influence appetite sensations, or concentrations of GIP\textsubscript{1-42} or total GLP-1, when fasted. Although some show high-calcium intake reduces appetite, these responses have, to date, been exclusively studied under energy-restriction\textsuperscript{11-14}. The present study provides evidence that calcium supplementation, may not influence appetite sensations (in the fasted state) under \textit{ad libitum} feeding, and therefore, might not result in spontaneous energy restriction. This should however, be confirmed by measures of energy intake under free-living conditions and with postprandial measures of appetite and metabolism, as nutritional status can influence appetite and metabolic responses to exercise\textsuperscript{40,41}. Ingestion of a high-calcium meal transiently enhances postprandial plasma GIP\textsubscript{1-42} and total GLP-1 concentrations\textsuperscript{7}. The present findings indicate that this does not result in upregulation of these peptides when fasted. Supporting this, others have reported that 1000 mg of calcium supplementation for 3 weeks does not influence fasting concentrations of total GLP-1 or GLP-1\textsubscript{1-36}\textsuperscript{42}, although postprandial concentrations were enhanced,
suggesting that the acute response that we previously reported\textsuperscript{7} translates into chronic adaptation manifest in the postprandial state.

An interesting finding was that fasting insulinemia was elevated by 2 weeks of calcium supplementation. Taken in aggregate with elevated HOMA-IR, this suggests reduced insulin sensitivity. These were not primary outcomes of the study and thus should be interpreted with caution.

Notwithstanding this, we previously reported elevated postprandial insulinemia following the consumption of a single high-calcium meal\textsuperscript{6,7}. Physiological hyperinsulinemia induces insulin resistance\textsuperscript{43}. Accordingly, it is not inconceivable that this transient elevation, which would likely occur following each CAL test drink ingested (relative to control) over the 14-d supplemental period, underlies the increase in insulinemia and HOMA-IR in the basal state. These findings conflict with some previous work whereby calcium supplementation improves\textsuperscript{44,45} or does not influence\textsuperscript{46} insulin sensitivity. These studies however, were performed in individuals at risk of, or diagnosed with, type 2 diabetes.

In 314 individuals randomized to calcium plus vitamin D supplementation for 3 y, those with impaired fasting glucose (5.6–6.9 mmol/L; $n = 92$) improved their insulin sensitivity (assessed by HOMA-IR), whereas those with normal fasting glucose showed no change. Therefore the effects of calcium
supplementation may need to be considered in context of the individual’s metabolic health. In a metabolically-healthy population, high calcium intake appears to have no benefit (and could possibly be detrimental) for insulin sensitivity. This effect of calcium supplementation on insulin resistance in healthy individuals should be confirmed by a postprandial measure, or a euglycemic hyperinsulinemic clamp. The interpretation of this finding should be taken with caution as pre-intervention insulin and HOMA-IR differed, and this was a secondary outcome. Serum insulin concentrations negatively correlated with $\dot{V}O_2$peak. This is not a novel finding, but provides support that the measures of insulin concentration and of oxygen uptake were valid on an inter-individual level.

There are some limitations with this study that deserve acknowledgement. The power calculation was based on the primary outcome of whole-body lipid utilization, therefore the blood-based and appetite variables should be interpreted with caution. The method of participant recruitment may have led to a degree of selection bias and, finally we cannot be certain that the CON period resulted in sufficient “calcium deficiency” to influence lipid utilization. Nevertheless, the dietary restrictions imposed (described in Supplementary Information 2), would likely result in a calcium intake approximating ~700 mg/d

(including the CON drink). Which, given that physically active individuals likely experience calcium losses during exercise,
it could be assumed that this produces a similar calcium
balance to Melanson et al.\textsuperscript{18}.

This is the first double-blind, randomized controlled study to investigate the effects of chronic (> 7 d) high-calcium intake on substrate metabolism during exercise. The blinding was effective, as evidenced by the incapability to correctly identify the test drinks. The supplement was independently verified, and increased calcium intake was not confounded by additional dairy components such as specific amino acids and fatty acids. In conclusion, two-weeks of calcium supplementation (~ 1000 mg/d) in physically active males, does not influence substrate metabolism during exercise or appetite sensations at rest, but does elevate fasting insulin concentrations in serum.

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Author contributions
Critically reviewed the paper: JTG, BPG, MDC, PLSR, EJS.

Conceived and designed the experiments: JTG, EJS. Performed the experiments: JTG, BPG, MDC. Analyzed the data: JTG, EJS. Wrote the paper: JTG, EJS.

Supplementary information is available at the European Journal of Clinical Nutrition website (http://www.nature.com/ejcn)

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Figure legends

Figure 1. Overview of the study design. CAL, high calcium; CON, control.

Figure 2. Change in serum insulin concentration pre- to post-supplementation with CON and CAL. CAL, high-calcium; CON, control. Individual data are presented with group means ± SEM represented by horizontal lines. n = 12. Paired t-test: *Significantly different to CON, P = 0.006.

Figure 3. Change in maximum rates of lipid utilization during exercise pre- to post-supplementation with CON and CAL. CAL, high-calcium; CON, control. Individual data are presented with group means ± SEM represented by horizontal lines. n = 13.

Figure 4. Plasma NEFA and glycerol concentrations during exercise before and after CON and CAL. a, NEFA concentrations with CON supplementation. b, NEFA concentrations with CAL supplementation. C, glycerol concentrations with CON supplementation. d, glycerol concentrations with CAL supplementation. CAL, high-calcium; CON, control; NEFA, non-esterified fatty acid. Data are mean ± SEM. n = 12. Interaction effect (supplement x time x power
output), $P = 0.883$ and $P = 0.401$ for NEFA and glycerol, respectively.