Hormonal Response to Prolonged Endurance Exercise in Elite Keto-Adapted Ultra-Endurance Athletes

Running Head: Endocrine Response in Keto-adapted Athletes

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Abstract

Objective: A burgeoning body of empirical and experimental evidence indicates that nutritional ketosis induced by carbohydrate restriction has clinical and physical performance applications. We recently reported that elite keto-adapted endurance athletes had greater than two-fold higher rates of fat oxidation, yet muscle glycogen at rest and after exercise was the same as a matched group of high-carbohydrate athletes. Given the importance of endocrine regulation of substrate mobilization and oxidation, the purpose of this study was to compare hormonal profiles to prolonged exercise in keto-adapted and high-carbohydrate athletes. **Methods:** Low Carbohydrate (n=10) and High Carbohydrate (n=10) ultra-endurance athletes, in the top 10% of race finishers, were enrolled for participation in this study. Participants completed body composition assessment via dual energy x-ray absorptiometry followed by a 180-min treadmill run at 64% VO2max. Venous blood sampling was obtained pre, during, and up to 2 hr post-exercise to assess testosterone, insulin-like growth factor 1, growth hormone, insulin like growth factor binding protein 1, glucagon, leptin and cortisol. Baseline hormone concentrations and body composition differences were assessed via independent t-tests. Biochemical responses to exercise and diet were assessed via a 2-way repeated measures analysis of variance. **Results:** No significant differences existed between groups in body composition. Resting plasma growth hormone concentrations in keto-adapted athletes were 3.5-fold higher ($P<0.05$). Glucagon concentrations were significantly higher in all but one timepoint for low carbohydrate athletes. A significant main effect of time, but not diet, was observed for testosterone, insulin-like growth factor 1, growth hormone, insulin like growth factor binding protein 1, and cortisol. Normalized testosterone and insulin like growth factor 1 demonstrated trends for higher concentrations in the low carbohydrate group post-exercise. **Conclusion**: The pronounced shift to lipid oxidation at rest and during submaximal exercise in keto-adapted athletes is accompanied by a differential endocrine response characterized by elevated growth hormone and glucagon and decreased leptin concentrations.

Key Words: Ketogenic, Athletes, Endocrinology, Metabolism

INTRODUCTION

The principle that carbohydrate is essential and should constitute the primary macronutrient in an athlete's diet is based on experimental data performed over 80 years ago [1] that was reinforced by pioneering researchers in the 1960s [2,3] and codified into contemporary sports nutrition guidelines that persist today [4]. In contradistinction to the long-standing dogma that a high-carbohydrate diet is optimal for all athletes, a burgeoning number of endurance athletes have abandoned carbohydrate-dense foods in favor of a low-carbohydrate, high-fat, and moderate-protein diet. Our research team recently studied the metabolic responses to exercise in 'keto-adapted' athletes who were competing at a high level in ultra-endurance running events and had been consuming a low-carbohydrate diet for at least 6 months [5]. Peak fat oxidation was two-fold higher in keto-adapted versus a matched group of runners consuming a high-carbohydrate diet, and nearly 90% of energy expenditure was derived from fat during a 3-hr exercise bout. There were also significant differences in circulating metabolites (e.g. beta-hydroxybutyrate, glycerol, lactate), but surprisingly skeletal muscle glycogen was not significantly different pre- and post-exercise.

The rationale for switching to a low-carbohydrate diet is based in part on the positive metabolic adaptations associated with accelerated ketogenesis and fatty acid oxidation described previously [5], which often manifests in greater ease of fat loss and improved body composition [6]. Recently identified non-metabolic functions attributed to beta-hydroxybutyrate, such as histone deacetylase inhibition and enhanced protection from oxidative stress [7] and anti-inflammatory signaling [8], may also confer phenotypic advantages that promote improved recovery and performance [9,10]. Reduced insulin stimulation in response to reduced carbohydrate intake drives the robust shift to near exclusive reliance on lipid fuel at rest and during submaximal exercise,

but other hormonal underpinnings that shape the keto-adapted phenotype have not been well described.

Dietary nutrients alter availability of metabolites and hormone concentrations that control body composition through their impact on adipose tissue lipolysis and skeletal muscle signaling targeting protein balance [11]. Lower stimulation of insulin in response to a ketogenic diet accelerates adipose tissue lipolysis and fatty acid oxidation [12], but may compromise anabolism in skeletal muscle if not offset by other anabolic/anti-catabolic hormones. There is a scarcity of research on anabolic (e.g., growth hormone, insulin-like growth factor-1, and testosterone) and catabolic (e.g., cortisol, glucagon) hormone profiles in keto-adapted athletes. The primary purpose of this study was to characterize the resting and exercise-induced hormonal patterns in elite ultra-runners habitually consuming either a low-carbohydrate/high-fat ketogenic or traditional high-carbohydrate diet.

METHODS

Experimental Design

A detailed experimental approach has been previously described [5]. In brief, this study compared two separate cohorts of elite ultra-endurance athletes from various regions of the United States habitually consuming either a high carbohydrate (HC) or low carbohydrate (LC) diet. Subjects completed two consecutive days of laboratory testing. On Day peak aerobic and fat oxidation capacity was determined on a treadmill. On Day 2 subjects completed a 3 hr treadmill run at 64% of VO_{2max}. Blood was taken before and after exercise to determine hormonal responses to exercise. Muscle biopsies were obtained for metabolomics analysis at baseline, immediately post exercise, and two hours post exercise.

Participants

As previously reported the LC (n=10) and HC (n=10) subjects were well matched in terms of training/competition status and physical characteristics (Table 1). Athletes were only considered for participation in the study if they were in the top 10% of finalists in sanctioned running races ≥50km, or triathlons ≥113km. Many of the athletes were ultra-endurance record holders (Course: 30%, National: 10%, International: 10%) and a quarter of the participants had made Team USA appearances. Screening questionnaires were completed by interested athletes to determine health history, diet, and eligibility. Further dietary screening was completed by a registered dietician via phone interview. In order to be eligible athletes had to have been habitually consuming either a LC (CHO: < 20% and Fat: > 60%) or HC (CHO: >55%) diet for a period of at least 6 months. Exclusion criteria consisted of failure to meet dietary requirements, health issues including, but not limited to, diabetes, heart disease, liver, kidney or other metabolic or endocrine dysfunction, injury, anabolic drug use or excessive bleeding. Prior to enrollment all participants signed an informed consent document approved by an Institutional Review Board.

Table 1. Descriptive Characteristics

	Low Carbohydrate Diet (n=10)	High Carbohydrate Diet (n=10)	P Value
Age, years	34.1 ± 7.1	32.9 ± 6.0	0.689
Height, cm	175.7 ± 7.8	173.9 ± 5.3	0.555
Body mass, kg	68.8 ± 8.2	66.5 ± 6.8	0.491
Body fat, %	7.8 ± 2.4	9.6 ± 4.3	0.266
Lean mass, kg	60.9 ± 7.1	57.3 ± 5.0	0.387
Fat mass, kg	5.5 ± 1.9	6.5 ± 3.3	0.207
VO_2max, L/min	4.46 ± 0.39	4.25 ± 0.46	0.299
VO_2max, mL/kg/min	64.7 ± 3.7	64.3 ± 6.2	0.85
Daily Calories	3174 ± 611	2884 ± 814	0.38
Protein (g)	118 ± 38	139 ± 32	0.186
Protein (daily %)	14.4 ± 3.5	19.4 ± 2.4	0.001
Carbohydrate, (g)	486 ± 128	82 ± 62	0
Carbohydrate (daily %)	59.1 ± 10.2	10.4 ± 4.9	0
Fat (g)	91 ± 31	226 ± 66	0
Fat (daily %)	25.0 ± 7.4	69.5 ± 6.0	0
Competitive running experience (years)	11 ± 8	9 ± 6	0.583

Note: All data presented as Mean ± SD

Submaximal Exercise Protocol

Prior to beginning the exercise a fasted blood sample was taken via venipuncture in the antecubital fossa and a skeletal muscle biopsy was obtained from the vastus lateralis. Subjects then consumed a shake (5 kcal/kg body mass) with a macronutrient distribution (%carbohydrate:fat:protein) of 5:81:14 in the LC group and 50:36:14 in the HC group to roughly mirror their habitual diet macronutrient composition. After consumption, participants sat quietly for 90 min, and then ran for 180 min on a treadmill at an intensity equal to 64% VO_{2max}. During exercise athletes were allowed to consume water ad libitum with no other nutritional input. The treadmill was briefly stopped at 60 and 120 min to allow for venipuncture from the antecubital fossa. Heart rate, RPE and indirect calorimetry were obtained as described in Figure 1.

Immediately following the treadmill run participants were seated in a wheelchair and blood samples were obtained. Participants were then relocated to a hospital bed for completion of the second muscle biopsy. After completion of the biopsy participants were given a second shake to consume that was identical to the earlier one. Blood samples were collected 30, 60 and 120 min post-exercise. A final muscle biopsy was obtained at two hours post exercise.

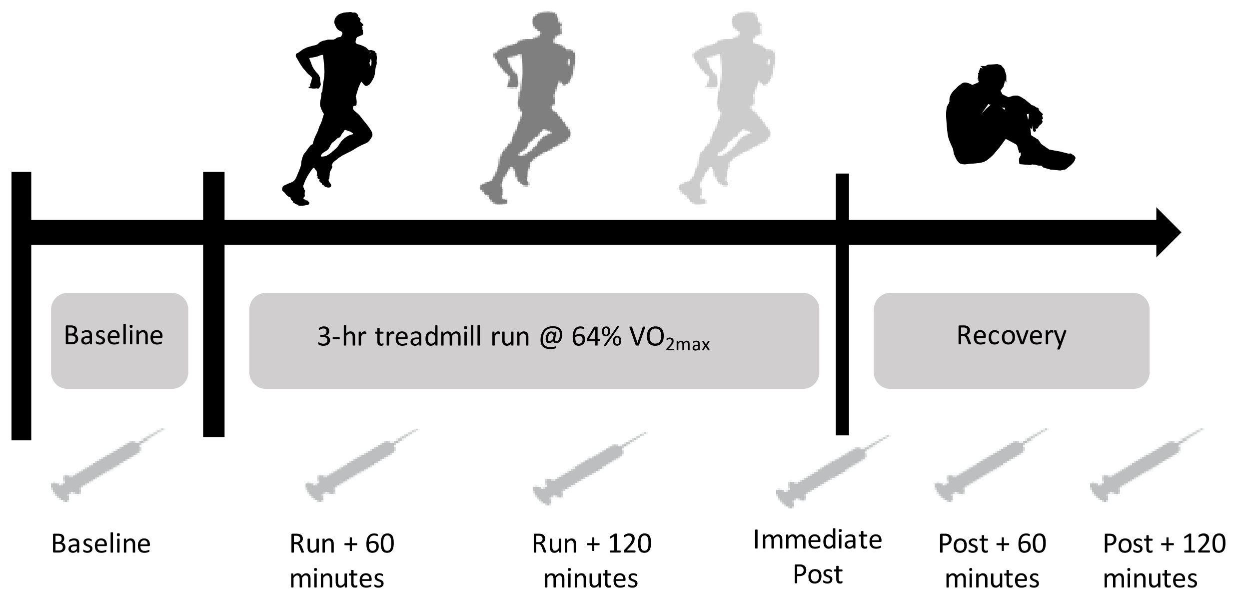

Figure 1. Schematic representation of the testing protocol.

Biochemical Analysis

Blood samples were collected from the antecubital fossa via indwelling Teflon cannula or 21-gauge butterfly needle. When used, indwelling lines were kept patent with saline solution flushes. Prior to subsequent draws a 3mL vacutainer of blood was collected and discarded to avoid collection of any saline solution. Blood was collected using appropriate tubes for analysis (i.e. serum and EDTA vacutainers). All blood was centrifuged at 4°C for 15 min at a speed of

1500 x g. Serum or plasma fractions were then aliquoted and stored at -80°C until batch analysis. Samples underwent a maximum of two freeze-thaws cycle prior to analysis.

Serum cortisol and testosterone were measured via enzyme-linked immunosorbent assay (ELISA) kit with an analytical sensitivity of 25 ng/mL and 0.05 ng/mL, respectively (Calbiotech, Spring Valley, CA). Glucagon (sensitivity: 6.37 pg/mL), IGF-l (sensitivity: 0.026 ng/mL), IGFBP1 (sensitivity: 13.8 pg/mL), leptin (sensitivity: <7.8 pg/mL), GH (22kDa) (sensitivity: 2.10 pg/mL) and SHBG (sensitivity: 0.006 nmol/L) were analyzed via Quantikine ELISA kits (R&D Systems, Minneapolis, MN) using a microplate reader (Gen5, Biotek Instruments, Winooski, VT). Coefficients of Variation (CV) for each of the analyzed hormones were less than 10% for both inter-assay and intra-assay plate reader analysis.

Statistical Analysis

Main effects of time and diet for all hormonal markers were determined using a multilevel model for factorial repeated measures. The Shapiro-Wilk and Levene's tests were used to evaluate assumptions for normality and constant variance, respectively ($\alpha=0.10$). When necessary, data transformations were used to meet both assumptions. Due to non-normal distribution of GH data, a logarithmic transformation was utilized. Pairwise comparisons between dietary groups were made at each time point using paired t-tests. A paired Wilcoxon rank-sum test was used in place of the t-test when transformation failed to correct lack of normality or constant variance. Correlations between hormonal markers and previously published metabolic markers [5] were determined based on Spearman's rank coefficient. Area under the curve analysis (AUC) was completed to determine differences in the total integrated hormone exposure. Additionally, due to the heterogenous nature of the testosterone and IGF1,

post-hoc analysis was completed with timepoint data normalized to baseline. Effect sizes were also calculated to determine the strength of differences between groups. All aforementioned analyses were performed using R 3.2.3 (R Core Team 2015).

RESULTS

Previously Reported Data

As reported previously [5], LC athletes consumed 10% of their energy intake from carbohydrate (82 g/day) compared to 59% of energy from carbohydrate (486 g/day) in HC athletes. Peak fat oxidation during the VO2max test and submaximal fat oxidation during the 3-hr treadmill run were two-fold higher in LC athletes. Serum ketones and glycerol were also approximately two-fold higher in LC athletes indicating accelerated ketogenesis and adipose tissue lipolysis. Skeletal muscle glycogen was not significantly different between LC and HC athletes before or after exercise.

Hormonal Responses

A main effect of diet was observed for glucagon ($p=0.022$) and GH ($p=0.033$). In both groups glucagon gradually increased during the 3 hr run, peaked immediately post-exercise, and returned to baseline at 120 min post-exercise (Fig 2). Glucagon was 1.4-fold higher at baseline in LC athletes, and this difference between groups persisted throughout exercise and recovery (1.4 to 1.7-fold higher in LC athletes). In both groups GH peaked 60 min into the run, stayed elevated throughout exercise, and returned toward baseline during recovery (Fig 2). Baseline GH concentrations were 3.5-fold higher at baseline in LC athletes, and remained higher throughout exercise relative to HC athletes.

A main effect of time was observed for all hormones including testosterone, cortisol, GH, glucagon, IGF-1, IGFBP-1, and leptin (p<0.000). There was a trend for an interaction between time and diet for IGFBP-1 (p=0.06), and GH (p=0.09). No significant differences were observed between dietary groups at any time point for IGF-1, IGFBP-1, leptin, or testosterone.

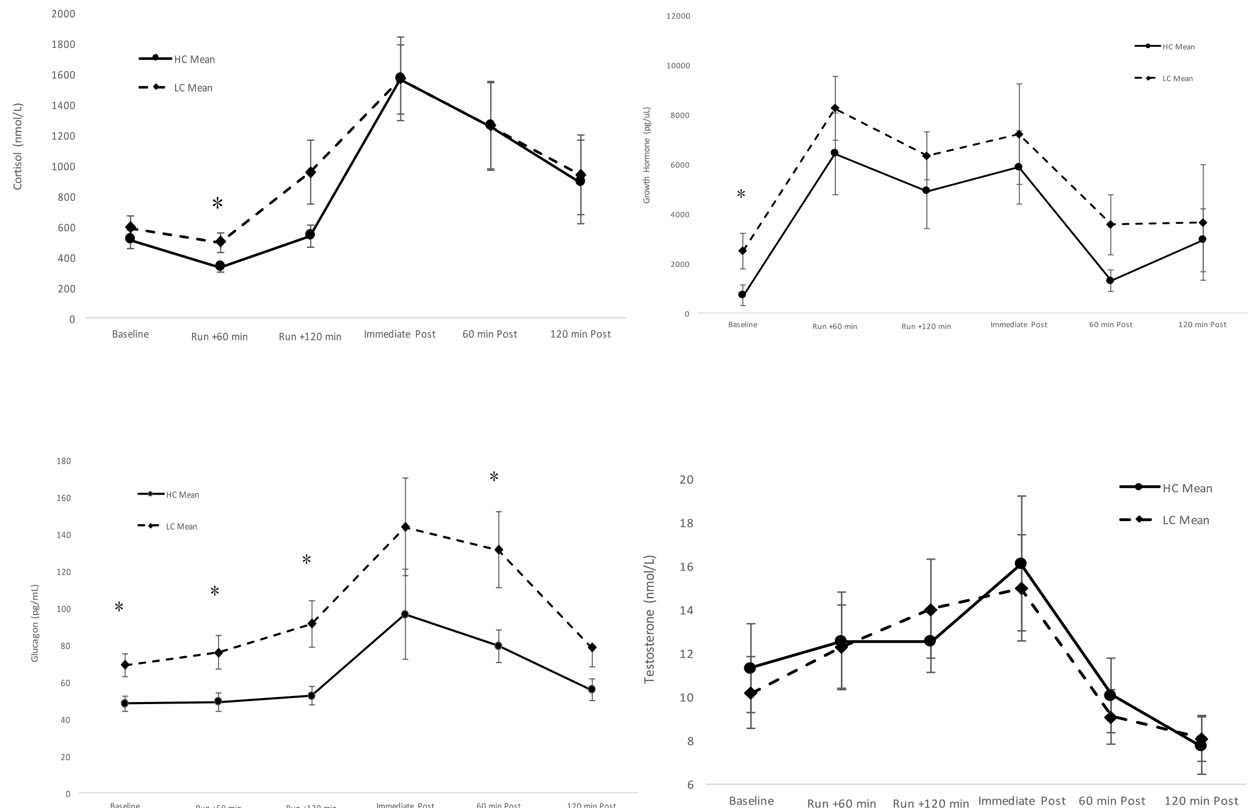

Fig 2. Cortisol (upper left), growth hormone (upper right), glucagon (lower left), and testosterone (lower right) responses to 3 hr of running in low-carbohydrate (LC) and high-carbohydrate (HC) athletes. There were significant main effects of time for all hormones and a significant group effect for growth hormone and glucagon. *Significantly (P<0.05) different than baseline.

Due to previous literature demonstrating correlations between levels of adiposity and leptin the current study sought to determine if any correlations existed after keto-adaptation. As

expected, leptin and adiposity were highly correlated (r=0.816) across the entire cohort. After adjustment for habitual diet consumed we found that leptin and adipose tissue mass are still highly correlated for both HC (r=0.86) and LC athletes (0.806). At rest, leptin concentrations were significantly lower in the LC athletes (p=0.019) conferring the potential of enhanced leptin sensitivity.

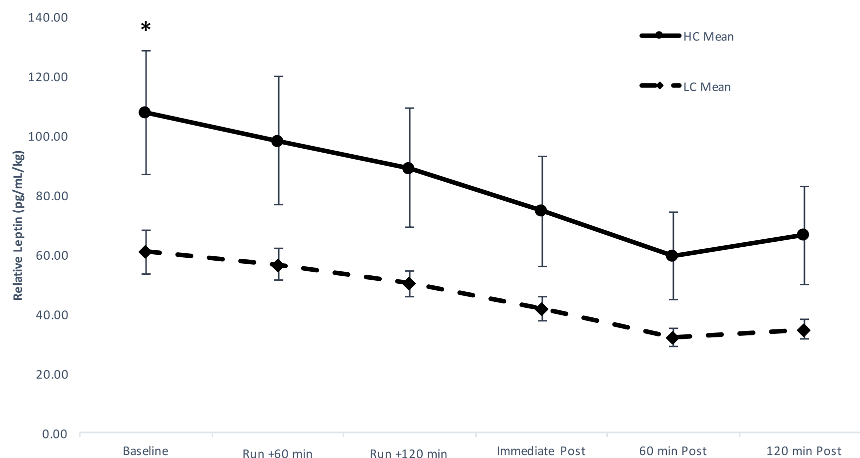

Fig 3. Leptin responses to 3 hr of running in low-carbohydrate (LC) and high-carbohydrate (HC) athletes. There were significant main effects of time. *Significantly (P<0.05) different than baseline.

Area under the curve analysis was completed to determine, if any, differences in hormone exposure existed. LC athletes had significantly greater concentrations of glucagon across the observed testing times ($p < 0.05$).

Normalization analysis failed to demonstrate significant differences between groups. A trend for significantly higher normalized testosterone in the LC group existed at 120 minutes post exercise (p=0.062: Cohen’s D = 0.9). Further, trends for higher IGF-1 in LC existed at both two hours of running and immediately post exercise (p=0.079, Cohen’s D= 0.83 and p=0.06, Cohen’s D= 0.9 respectively).

DISCUSSION

The hallmark of keto-adaptation is a dramatic shift to greater fat oxidation at rest and during submaximal exercise in both the post absorptive and postprandial state. The pronounced shift to enhanced lipid oxidation associated with nutritional ketosis occurs in a wide range of individuals from obese insulin resistant type-2 diabetics to highly trained elite endurance athletes [5], indicating the process of keto-adaption is highly evolved and conserved. In elite endurance athletes, the higher absolute rates of oxygen uptake and ATP turnover make it an attractive model to study other aspects of keto-adaptation. Given the importance of endocrine signaling in regulating metabolism at all levels from transcription to translation, we set out to determine if there were differential hormonal responses between keto-adapted and high-carbohydrate athletes. Our results indicate that a differential hormonal milieu exists peri-exercise in keto-adapted ultra-endurance athletes characterized by increased growth hormone and glucagon, and decreased leptin concentrations.

Keto-adapted athletes showed higher GH levels, which were most pronounced in the fasted stated as evidenced by 3-fold higher pre-exercise concentrations. There was wide variation between athletes, especially GH concentrations during the 3 hr run, which may be partially attributable to the pulsatile nature of GH. Recent findings have demonstrated that athletes may have significantly greater GH concentrations prior to exercise compared to sedentary controls and thus may represent a positive exercise anticipatory adaptation prior to long-distance running [13]. Functionally, GH has anabolic/anti-catabolic effects on skeletal muscle protein [14], but it also stimulates adipose tissue lipolysis [15] manifesting in potent nutrient partitioning effects (i.e., increased lean body mass and decreased fat mass) [16]that could account for the slightly leaner phenotype observed in LC (7.8% body fat) vs HC (9.6% body fat) athletes. The anabolic effects

of GH may contribute to maintenance of adequate muscle mass in LC athletes, and the lipolytic effects of GH may complement lower insulin signaling to account for the two-fold higher rates of fat oxidation observed in these athletes [5]. The molecular details are poorly understood, but the lipolytic effects of GH appear to be modulated via beta-adrenergic receptors with a slightly greater effect attributed to the 22 kD than the 20 kD variant [17]. Lipolytic effects of GH appear to be attributed to a 15 amino acid C-terminal fragment that has since been synthesized and studied as an anti-obesity drug [15,18]. Differential secretions of the GH superfamily following acute endurance exercise has been previously reported [19]. Wallace et al. [19] hypothesized that the diminished secretion/increased clearance of 22kDa monomer and the concomitant increase of 20kDa and 17kDa monomers may be a compensatory mechanism to prevent post-exercise hypoglycemia. These finding may help to explain how keto-adapted athletes are able to maintain glycaemia post exercise in the context of low exogenous carbohydrate availability [20].

As expected we observed a significant increase in GH post-exercise but the response was greater than expected considering GH is more responsive to higher-intensity exercise. Moreover, we expected the GH response to exercise to be attenuated in LC athletes since high-fat feeding prior to exercise reduces the exercise-induced GH response compared to carbohydrate or fasting [21,22], which may be due to elevations in circulating fatty acids that inhibit GH release [23]. The preserved GH response after a high-fat meal indicates that chronic keto-adaption encompasses changes in the metabolic stimuli that regulate GH responses to exercise. It is important to note that antigen capture assays, such as the ELISA methods used in the current study, only measure concentration of the 22kDa isoform of GH due to differences in binding epitopes. Although the molecular details linking nutritional ketosis and the anterior pituitary require elucidation, our results provide evidence for a specific effect of keto-adaptation on 22 kD GH.

Several cohort studies demonstrate an inverse relationship between dietary carbohydrate, insulin sensitivity and circulating IGF1 [24,25]. Insulin-like growth factor 1 (IGF1) is a polypeptide hormone primarily secreted from the liver in response to GH release. Despite the shared pathways between IGF1 and the glucose-insulin axis, macronutrient composition of meals peri-exercise exert minimal effects on [IGF1] [26-28]. Previous research has demonstrated that different GH variants may differentially mediate hepatic output of IGF1, with 22kDa possessing a greater effect than 20kDa [29]. Both athlete groups had a significant effect of time on IGF1 as a result of the prolonged endurance exercise stimulus. The current study demonstrates however that despite time-dependent differences in 22kDa GH secretion no differences in [IGF1] were observed. This finding may represent an altered pituitary regulation of GH variants when keto-adapted.

Leptin has long been known to be an adipokine, secreted by adipose tissue. Although its most commonly recognized function is appetite suppression, it also stimulates fatty acid oxidation. Given the much greater rate of peak fat oxidation in keto-adapted athletes [5], we anticipated a corresponding elevation in leptin. We found that, relative to adipose tissue mass, LC athletes had significantly less circulating leptin at baseline. This is in line with previous research on ketogenic diets that have shown decreased leptin concentrations, an indicator of leptin sensitivity [30]. Leptin's influence on fat oxidation appears, at least in part, to result from antagonism of insulin [31], possibly through reduced binding in adipocytes [32]. Additionally, leptin has been demonstrated to regulate appetite via AMPK mediated pathways [33].

Despite LC athletes consuming significantly greater amounts of dietary fat and cholesterol[5] which has been associated with higher circulating testosterone, no significant differences were observed in regards to resting or exercise-induced total testosterone. One of the primary anabolic responses to skeletal muscle damage, including mechanical and metabolic

strain as a result of marathon running, is increased secretion of testosterone. Importantly, testosterone exerts effects non-preferentially and can enhance anabolism in both Type I and Type II fibers. This demonstrates that despite consuming a fraction of the carbohydrate per day of the HC group, the LC group had no differences in circulating testosterone. If sample sizes were larger, it is possible that the recovery T values for LC may have been higher.

Cortisol, a glucocorticoid hormone produced in the adrenal glands is produced in response to long-term, chronic stressors as well as high intensity exercise [34]. Previous research has shown that 65% VO_{2max} may not be a sufficient stressor to induce significant cortisol changes [34]. At the onset of exercise when energy needs outweigh the energy production capabilities, the increased cortisol secretion (or dampened clearance) may induce hepatic gluconeogenesis to provide energy substrate. Research by Webster et al. [20] demonstrated in LC cyclists completing a two-hour exercise trial at 55% peak power output had no significant differences in absolute gluconeogenesis rates, but LC athletes had a higher percentage of endogenous glucose production from gluconeogenesis. However, once lipid oxidation rates reach elevated rates, enough ATP is available for further fueling. This may help explain how differences are not noted at baseline, Run +120 minutes or post exercise.

Glucagon is a peptide hormone released by the alpha cells of the pancreas. Like cortisol, glucagon stimulates catabolism of endogenous energy stores in the presence of low blood glucose concentrations. In humans, glucagon infusion is known to increase circulating levels of free fatty acids, glycerol, and β-hydroxybutyrate [35], all indicative of increased lipolysis. Consistent with this response, glucagon was higher in keto-adapted athletes at nearly all timepoints and correlated with glycerol, β-hydroxybutyrate, and total ketones. The consistently higher glucagon levels are therefore likely to have contributed, possibly in conjunction with GH,

to the greater peak fat oxidation in keto-adapted athletes [5]. Furthermore, given the role of glucagon and cortisol in gluconeogenesis, the elevation of these two hormones may have contributed to the keto-adapted athletes' ability to maintain glycogen levels similar to their non-keto-adapted counterparts [5]. These findings warrant further investigation into the role of glucagon, cortisol, and growth hormone in keto-adaptation.

Conclusion

Ketoadaptation is associated with dramatic alterations in substrate use in obese insulin resistant populations, and this is now evident in lean highly trained ultra-endurance athletes. We can now extend these metabolic adaptations to include differential endocrine responses, most notably increased GH and glucagon concentrations. Further research on hormone signaling and down-stream targets will help shed light on the potential athletic and therapeutic benefits associated with the keto-adapted phenotype.

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