ORIGINAL ARTICLE

JBMR

Glucocorticoid-Induced Insulin Resistance in Men Is Associated With Suppressed Undercarboxylated Osteocalcin

Lewan Parker,[1,2] Xuzhu Lin,[2] Andrew Garnham,[1,2] Glenn McConell,[2] Nigel K Stepto,[2,3,4] David L Hare,[5] Elizabeth Byrnes,[6] Peter R Ebeling,[7] Ego Seeman,[8,9] Tara C Brennan-Speranza,[10] and Itamar Levinger[2,3,5]

[1]Institute for Physical Activity and Nutrition (IPAN), Deakin University, Geelong, VIC, Australia
[2]Institute of Health and Sport (IHES), Victoria University, Melbourne, VIC, Australia
[3]Australian Institute for Musculoskeletal Science (AIMSS), Department of Medicine-Western Health, Melbourne Medical School, The University of Melbourne, Melbourne, VIC, Australia
[4]Monash Centre of Health Research and Implementation (MCHRI), School of Public Health and Preventative Medicine, Monash University, Melbourne, VIC, Australia
[5]University of Melbourne and the Department of Cardiology, Austin Health, Melbourne, VIC, Australia
[6]PathWest QEII Medical Centre, Perth, WA, Australia
[7]Department of Medicine, School of Clinical Sciences, Monash University, Melbourne, VIC, Australia
[8]Department of Endocrinology, Austin Health, University of Melbourne, Melbourne, VIC, Australia
[9]Mary Mackillop Institute of Health Research, Australian Catholic University, Melbourne, VIC, Australia
[10]Department of Physiology, University of Sydney, Sydney, NSW, Australia

ABSTRACT

In mice, glucocorticoid-induced insulin resistance occurs largely through impaired osteoblast function and decreased circulating undercarboxylated osteocalcin (ucOC). Whether these mechanisms contribute to glucocorticoid-induced insulin resistance in humans has yet to be established. In addition, the effects of glucocorticoids on the exercise-induced increase in circulating ucOC and insulin sensitivity are also unknown. We hypothesized that acute glucocorticoid treatment would lead to basal and postexercise insulin resistance in part through decreased circulating ucOC and ucOC-mediated skeletal muscle protein signaling. Nine healthy men completed two separate cycling sessions 12 hours after ingesting either glucocorticoid (20 mg prednisolone) or placebo (20 mg Avicel). The homeostatic model assessment was used to assess basal insulin sensitivity and a 2-hour euglycemic–hyperinsulinemic clamp was commenced 3 hours after exercise to assess postexercise insulin sensitivity. Serum ucOC and skeletal muscle protein signaling were measured. Single-dose glucocorticoid ingestion increased fasting glucose (27%, $p < 0.01$) and insulin (83%, $p < 0.01$), and decreased basal insulin sensitivity (−47%, $p < 0.01$). Glucocorticoids reduced insulin sensitivity after cycling exercise (−34%, $p < 0.01$), reduced muscle GPRC6A protein content (16%, $p < 0.05$), and attenuated protein phosphorylation of $mTOR^{Ser2481}$, Akt^{Ser374}, and $AS160^{Thr642}$ (59%, 61%, and 50%, respectively; all $ps < 0.05$). Serum ucOC decreased (−24%, $p < 0.01$) which correlated with lower basal insulin sensitivity ($r = 0.54$, $p = 0.02$), lower insulin sensitivity after exercise ($r = 0.72$, $p < 0.05$), and attenuated muscle protein signaling ($r = 0.48$–0.71, $p < 0.05$). Glucocorticoid-induced basal and postexercise insulin resistance in humans is associated with the suppression of circulating ucOC and ucOC-linked protein signaling in skeletal muscle. Whether ucOC treatment can offset glucocorticoid-induced insulin resistance in human subjects requires further investigation. © 2018 American Society for Bone and Mineral Research.

KEY WORDS: GLUCOCORTICOID METABOLISM; ANTI-INFLAMMATION; GLYCEMIC CONTROL; HIGH-INTENSITY INTERVAL EXERCISE; INSULIN SIGNALING

Received in original form April 16, 2018; revised form August 5, 2018; accepted August 8, 2018. Accepted manuscript online August 23, 2018.
Address correspondence to: A/Prof Itamar Levinger, Victoria University, PO Box 14428, Melbourne, VIC 8001, Australia. E-mail: itamar.levinger@vu.edu.au
Public clinical trial registration: www.anzctr.org.au, ACTRN12615000755538. The Effects of Acute Exercise on Insulin Sensitivity, Bone Metabolism and Cardiovascular Risk Factors in Healthy Young Men Treated With a Single Dose of Prednisolone
Additional Supporting Information may be found in the online version of this article.

Journal of Bone and Mineral Research, Vol. 34, No. 1, January 2019, pp 49–58
DOI: 10.1002/jbmr.3574

Introduction

Osteoblasts synthesize and secrete noncollagenous proteins including osteocalcin (tOC). The undercarboxylated form of osteocalcin (ucOC) is reported to regulate insulin secretion and sensitivity in mice.[1,2] For example, ucOC-deficient mice are insulin resistant, whereas treatment of obese mice with ucOC restores insulin secretion and sensitivity and enhances insulin-stimulated glucose uptake following ex vivo muscle contraction.[1,3,4] In humans, ucOC may also regulate glycemic control and postexercise insulin sensitivity.[5–9] However, evidence for this relies on cross-sectional and observational studies.[10] The daily control of glucose and insulin is important for the prevention and management of type 2 diabetes and other cardiometabolic conditions.[11] As such, further exploration of potential novel targets that mediate glycemic control, including the mechanisms behind the postexercise enhancement of insulin sensitivity, is warranted.

Insulin resistance associated with acute glucocorticoid (GC) treatment is reported to occur largely through the suppression of osteocalcin secretion rather than canonical GC signaling in the liver or skeletal muscle.[12,13] In humans, acute GC ingestion decreases both insulin sensitivity[14] and circulating ucOC,[15] but whether there is a causal relationship between these traits has yet to be established. In addition, acute exercise increases circulating ucOC, which is associated with improved postexercise insulin sensitivity in humans[7] and is reported to play an important role in skeletal muscle function and adaptation to exercise training in mice.[16,17]

GC treatment and exercise are also associated with changes in circulating bone remodeling markers including β-isomerized C-terminal telopeptides (β-CTx) and procollagen 1 N-terminal propeptide (P1NP),[14,16] which may also influence insulin sensitivity. However, this remains largely unexplored in humans.

In rodents, the ucOC-mediated glucose uptake signaling pathway in skeletal muscle includes the G protein-coupled receptor family C group 6-member A (GPRC6A) as the receptor with downstream activation of protein signaling pathways involving AMP-activated protein kinase (AMPK), protein kinase B (Akt), mechanistic target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK), Akt substrate 160 (AS160), and/or increased circulation of interleukin-6 (IL-6).[10,16,18,19] The role of these proteins in GC-induced insulin resistance and/or ucOC-mediated glycemic control in humans remains unclear. Furthermore, whether ucOC mediates postexercise enhancement of insulin sensitivity via these protein signaling pathways is unknown. We hypothesized that acute GC administration would suppress circulating ucOC, leading to a decrease in insulin sensitivity at rest and after exercise by compromising ucOC-associated muscle protein signaling.

Materials and Methods

Screening

The study protocol has been published.[20] A schematic overview of the project protocol and the consort flow chart are provided in Fig. 1 and Supplemental Fig. 1, respectively. Men were specifically recruited to avoid the potential confounding effects of sex on glucose and bone metabolism. Seventeen men were initially screened for eligibility. Exclusion criteria for participation included men with osteoporosis, metabolic or cardiovascular disease, and/or those taking medication known to affect bone metabolism, insulin secretion, or insulin sensitivity. Men with musculoskeletal and/or orthopedic conditions (such as severe osteoarthritis) that prevented normal daily function, or were undergoing medication or supplementation that could affect research outcomes such as glucocorticoids, warfarin therapy, or vitamin K, were also excluded from participation. Two participants did not meet the inclusion criteria and 4 declined to participate. Two participants voluntarily withdrew from the study prior to completion. Nine young men that were recruited from the general public completed the randomized, double-blinded, crossover study.

Participants were asked to refrain from physical activity (48 hours), and alcohol and caffeine ingestion (24 hours) prior to all testing sessions. Eligible participants provided a fasting blood sample that was analyzed at Austin Health Pathology (Melbourne, Australia) for glucose, HbA1c, and insulin. Body composition was analyzed via DXA (Software version 9.1; GE Lunar Prodigy, Madison, WI, USA) to assess body fat, lean body mass, fat mass in the abdominal region, and BMD to exclude osteoporosis.

On a separate day, participants completed a graded exercise test (GXT) on a cycle ergometer (Lode Excalibur Sport; Lode Medical Technology, Groningen, The Netherlands) to measure peak cycling aerobic capacity (VO_{2peak}), maximal power output during the VO_{2peak} test (W_{max}), and heart rate peak (HR_{peak}). The GXT protocol consisted of 1-min cycling stages starting at 20 W, which increased by 20 W every min until participants were unable to maintain a cycling cadence of 60 rpm or greater. Inspired and expired gas was analyzed via indirect calorimetry (Quark CPET; Cosmed, Rome, Italy). The W_{max} and HR_{peak} obtained during the GXT were used to calculate the workload for the main exercise sessions.

Experimental phase

After screening, participants underwent two identical sessions, with the exception of either prior placebo or GC ingestion, which included a single session of exercise, 3 hours of rested recovery, concluding with a 2-hour euglycemic–hyperinsulinemic clamp.

Twenty-four hours prior to their first session, participants completed a food diary that was replicated prior to subsequent sessions. Participants arrived in the laboratory in the morning after an overnight fast. A resting muscle biopsy and blood sample was taken; then participants performed a single session of high-intensity interval exercise (HIIE) on a cycle ergometer. The HIIE included a 6-min warm-up at 50% to 60% HR_{peak}, followed by 4×4-min cycling intervals at 90% to 95% HR_{peak}, interspersed with 2-min active recovery periods at 50% to 60% HR_{peak}. The workloads (W) for both exercise sessions were adjusted accordingly to achieve the participants targeted HR, and thus maintain the same relative exercise intensity between sessions. The target HR for the exercise sessions was determined by the Karvonen heart rate reserve method: exercise target HR = (% of desired exercise intensity $\times$ ($HR_{peak} - HR_{rest}$)) + resting HR_{rest}. After completing the exercise, participants recovered for 3 hours on a bed, after which a 2-hour euglycemic–hyperinsulinemic clamp (clamp) was performed. Muscle biopsies were also taken prior to the clamp (3 hours postexercise) and after 2 hours of the clamp. Blood samples were taken immediately; 30 min; 1, 2, and 3 hours postexercise; and 1.5 and 2 hours during the clamp.

The procedures for the two experimental sessions were identical, with the exception that participants orally ingested a capsule of placebo (Avicel-microcrystalline cellulose NF PH105)

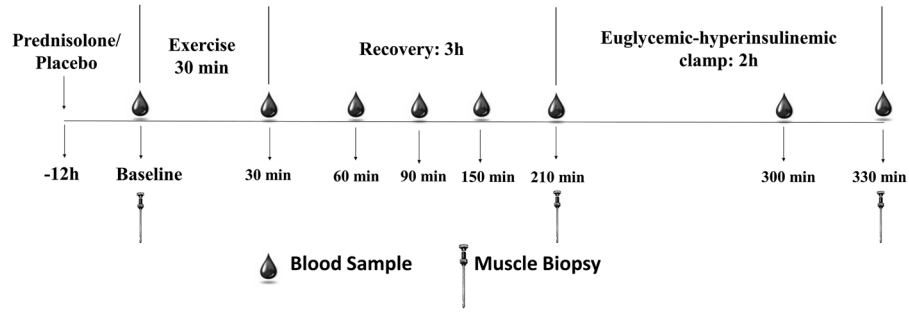

Fig. 1. Schematic depiction of the experimental design adapted from Levinger and colleagues.[20] Participants performed the indicated protocol twice, with either prior ingestion of GC (prednisolone) or placebo, orally in a double-blind, randomized, crossover design. The 2 experimental days were separated by a minimum of 1 week.

or glucocorticoid (20 mg of prednisolone), 12 hours prior to the commencement of exercise. The timing of ingestion was based on previously published data on the suppressive effect of acute GC treatment on osteocalcin in humans, which was confirmed during pilot testing in our own laboratory (data not shown).[15] The order of GC or placebo was randomly allocated in a double-blind fashion, using a block randomization model and sealed opaque envelopes. The randomization was completed by a university staff member independent of the research and research team. All researchers remained blinded until data analysis was complete. Both the GC and placebo were compounded by Thompsons Pharmacy (VIC, Australia). The 2 experimental days (GC and placebo) were performed 1 to 3 weeks apart to ensure that the effects of acute GC ingestion and high-intensity exercise were "washed out."[21,22]

Euglycemic–hyperinsulinemic clamp

The clamp was performed as previously reported.[7] A single slow-release tablet of potassium chloride (600 mg) was taken prior to the clamp to minimize the risk of potassium depletion. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at 40 mU/m^{-2}/min for 120 min generating an elevated, stable insulin concentration in the last 30 min of the clamp with no difference between trials (placebo: 498 ± 24 pmol/L; GC: 507 ± 15 pmol/L, $p < 0.01$ compared with baseline levels, $p = 0.56$ between trials). Insulin sensitivity was determined by the average glucose infusion rate (GIR; mg/kg^{-1}/min^{-1}) during the last 30 min of the insulin-stimulated period (M value) and the GIR expressed relative to per unit of insulin (M/I value).[23] Exogenous glucose was variably infused throughout the clamp to achieve a target blood glucose of ~ 5 mmol/L, which was assessed every 5 min from arterialized blood.

Skeletal muscle and blood sampling

Muscle samples were obtained from the vastus lateralis under local anesthesia (xylocaine 1%; AstraZeneca, Macquarie Park NSW 2113, Australia) utilizing a Bergström needle with suction.[24] The samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Venous blood was collected from an antecubital vein via an intravenous cannula, with collection tubes containing ethylenediaminetetraacetic acid (EDTA) or clot activator and serum gel separator. Blood samples were than separated into plasma/serum by centrifugation (10 min at 3500 rpm, 4°C) and immediately aliquoted and stored at −80°C until analyzed.

Biochemical analysis

Whole blood was analyzed immediately for blood glucose and lactate using an automated analysis system (YSI 2300 STAT Plus Glucose & Lactate Analyzer; YSI, Inc., Yellow Springs, OH, USA). Blood was analyzed at Austin Health Pathology using the standard protocols for HbA1c and insulin. Serum tOC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics, Mannheim, Germany). Serum ucOC was measured by the same immunoassay after adsorption of carboxylated OC on 5 mg/mL hydroxyl-apatite slurry, following the method described by Gundberg and colleagues.[25] Serum β-isomerized C-terminal telopeptides (β-CTx, a bone resorption marker) and procollagen 1 N-terminal propeptide (P1NP) were analyzed at Austin Health Pathology using a Roche Hitachi Cobas e602 immunoassay analyzer, according to the manufacturer's guidelines. IL-6 concentrations in serum were analyzed using a commercially available ELISA kit (Product #ab46042, Abcam, Cambridge, UK) as per the manufacturer's instructions. Serum IL-6 data for one participant were found to be a statistically significant outlier ($p < 0.05$; extreme studentized deviate test) and thus was excluded from IL-6 statistical analysis and correlations ($n = 8$). Importantly, exclusion of these data does not alter the main outcomes or conclusions of this study. Insulin resistance was estimated using the homeostatic model assessment (version 2) for insulin resistance (HOMA2-IR) using the Oxford Diabetes Trials Unit calculator (https://www.dtu.ox.ac.uk/homacalculator; University of Oxford, UK).

Table 1. Participant Characteristics

Age (years)	28 ± 2
Height (cm)	181 ± 3
Weight (kg)	80 ± 4
BMI (kg/m^{-2})	24 ± 1
W_{max} during GXT (Watts)	282 ± 18
HR_{peak} during GXT (BPM)	174 ± 4
VO_{2peak} (mL/kg^{-1}/min^{-1})	46 ± 3
HbA1c (%)	5.2 ± 0.1

Values are mean ± SEM. $N = 9$.

GXT = graded exercise test; W_{max} = maximum watts achieved during the GXT; HR_{peak} = peak heart rate measured during the GXT; VO_{2peak} = peak estimated volume of oxygen utilization measured during the GXT.

Table 2. The Effect of Glucocorticoids on Resting and Postexercise Glycemic Control

Variable	Placebo	Glucocorticoid
Resting measures of glycemic control		
Fasting glucose (mmol/L)	4.4 ± 0.1	5.6 ± 0.2*
Fasting insulin (pmol/L)	45.5 ± 5.8	83.1 ± 9.2*
HOMA2-IR	0.8 ± 0.1	1.6 ± 0.2*
Postexercise insulin sensitivity		
M value (mg/kg/min)	8.1 ± 0.5	5.5 ± 0.6*
M/I value (mg/kg/min/mU/L × 100)	11.5 ± 0.8	7.5 ± 0.7*

Values are mean ± SEM. $N = 9$. Two-tailed paired t tests were conducted on variables to detect significant differences between placebo and glucocorticoid administration.

M value = Average glucose infusion rate (mg/kg/min) during the last 30 minutes of the insulin-stimulated period; M/I value = The average glucose infusion rate expressed relative to per unit of insulin; HOMA2-IR = the homeostatic model assessment of insulin resistance version 2.

*Significantly different to placebo ($p < 0.01$).

Skeletal muscle protein analysis

Phosphorylation and abundance of specific proteins in whole muscle lysate were determined with all constituents present (ie, no centrifugation), to avoid the potential loss of total cellular protein that can occur with centrifugation.[26,27] Whole muscle lysate was analyzed as previously reported.[28] In brief, 30 cryosections of skeletal muscle (20 μm) were homogenized using a TissueLyser II (QIAGEN, Hilden, Germany) in RIPA lysis and extraction buffer [Cell Signaling Technology #9806 (Cell Signaling Technology [CST], Beverly, MA, USA): 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na3VO4, 1 μg/mL leupeptin] with 100mM dithiothreitol and 0.1% v/v protease and phosphatase inhibitor cocktail added (Sigma-Aldrich #P8340 and #P5726; Sigma-Aldrich, St. Louis, MO, USA). Homogenization was followed by gentle rotation at 4°C for 1 hour. Total protein content of muscle lysate was determined using the commercially available Bio-Rad protein assay and Bradford method as per the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Twelve μg of protein was prepared in Laemmli sample buffer (Bio-Rad), heated for 5 min at 95°C and separated by electrophoresis in 10% Criterion TGX Stain-Free Pre-Cast Gels (Bio-Rad). Separated proteins were transferred to a polyvinylidene difluoride membrane using a Trans-Blot Turbo Transfer System (Bio-Rad) and subsequently blocked with Tris-buffered saline-tween (TBST) and 5% skim milk for 1 hour at room temperature. Membranes were washed (3 × 5 min) with TBST and incubated at 4°C overnight with the following primary antibodies: phospho-ERK (Thr202/Tyr204; CST #9101), ERK (CST #9102), phospho-mTOR (Ser2481; CST #2974), mTOR (CST #2972), phospho-AMPKα (Thr172; CST #2531), AMPKα (CST #2532), phospho-IRS-1 (Ser307 in human; CST #2384), phospho-AS160 (Ser588, Ser318, Thr642; CST #8730, CST #8619, CST #4288, respectively), AS160 (CST #2447), phospho-Akt (Ser473; CST #9271), Akt (#9272), IRS-1 (Millipore #06-248; Millipore, Billerica, MA, USA), and GPRC6A provided by AVIVA (San Diego, CA, USA). After incubation, membranes were washed with TBST and incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase conjugated secondary antibody in 5% skim milk. Membranes were rewashed and incubated in SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min prior to imaging with a ChemiDoc Imaging System (Bio-Rad). All densitometry values are expressed relative to a pooled internal standard and normalized to the total protein content (densitometry) of each sample lane obtained from the stain-free image. There were no significant main or interaction effects for total protein content of IRS1, AS160, Akt, mTOR, and ERK (all $ps > 0.1$; data not shown). As such, phosphorylated proteins are expressed relative to antibody-specific total protein content.

Statistical analysis

Data were checked for normality and analyzed using Predictive Analytics Software (PASW v20; SPSS Inc., Chicago, IL, USA). Paired t tests were conducted to compare measures of insulin sensitivity and the percent increase in insulin-stimulated protein phosphorylation between placebo and GC ingestion trials. Comparisons of multiple means were examined using a 2-factor (capsule ingestion × time point) repeated measures analysis of variance. For all significant interaction and main effects, a priori comparisons of means (baseline versus all postexercise time points; placebo versus glucocorticoid for all time points) were conducted using Fisher's least significant difference test ($p < 0.05$). Spearman's rank correlation coefficients were determined to evaluate correlations between measures of glycemic control, bone remodeling markers, and skeletal muscle protein signaling. Data are reported as mean ± SEM; statistical analyses were conducted at the 95% level of significance ($p \leq 0.05$).

Study approval

This study was approved by, and conducted in accordance with, the Victoria University Human Research Ethics Committee (HRE14-099) and was registered with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au; ACTRN12615000755538). Verbal and written explanations about the study were provided prior to obtaining written informed consent.

Results

Glucocorticoids decrease tOC, ucOC, and insulin sensitivity

Participant characteristics are reported in Table 1. Compared with placebo, GC decreased basal and postexercise insulin sensitivity, and decreased serum tOC and ucOC (Table 2; Fig. 2*A*, *B*). The degree of suppression of tOC and ucOC correlated with the deterioration in basal and postexercise insulin sensitivity (Table 3). GC suppressed serum P1NP and increased serum β-CTx during the clamp (Fig. 2*C*, *D*, respectively); changes that were not associated with insulin sensitivity (all $ps > 0.1$; data not shown).

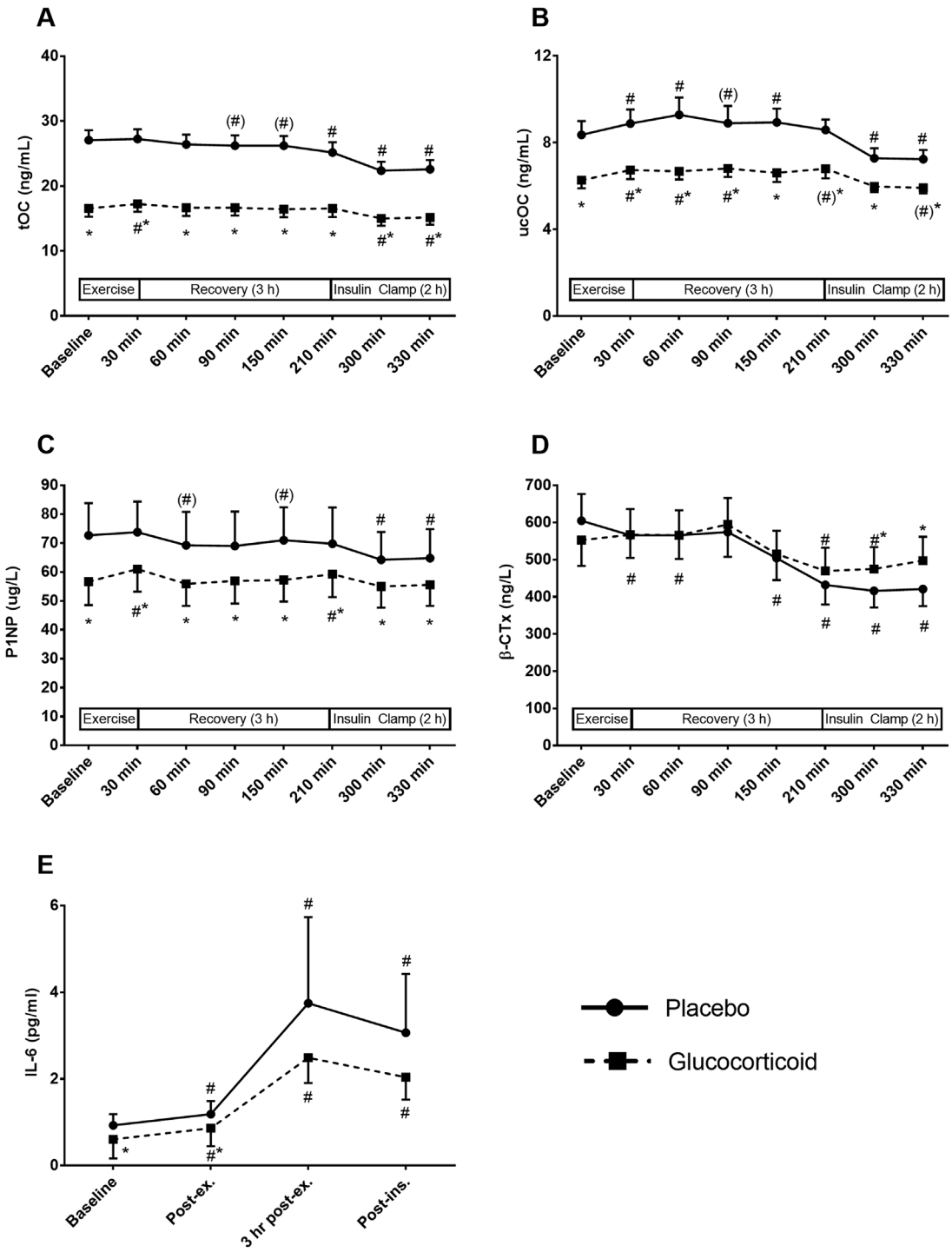

Fig. 2. The effect of glucocorticoid (prednisolone) on serum osteocalcin at baseline, postexercise, and postclamp (after insulin and glucose infusion). (*A*) Total osteocalcin (tOC), (*B*) undercarboxylated osteocalcin (ucOC), (*C*) procollagen type 1 N-terminal propeptide (P1NP), (*D*) beta C-terminal telopeptide of type 1 collagen (β-CTx), and (*E*) interleukin-6 (IL-6). Comparisons of multiple means were examined using a 2-factor (capsule ingestion × time point) repeated measures analysis of variance. A priori comparisons (baseline versus all postexercise time points; placebo versus GC for all time points) were conducted for interaction and main effects ($p \leq 0.1$) using Fisher's least significant difference. * = Significant difference ($p < 0.05$) compared with placebo trial; # = significant difference ($p < 0.05$) compared with baseline. Symbols in parenthesis are $p < 0.1$; $N = 8$ for IL-6 analysis; $N = 9$ for all other analyses. All data are presented as mean ± SEM.

Table 3. Spearman Rank Correlation Coefficients Between Measures of Glycemic Control, Skeletal Muscle Protein Signaling, and Serum Biomarkers at Baseline and After PostExercise Insulin Stimulation

	Resting/basal		Postexercise insulin stimulation		
	tOC	ucOC	tOC	ucOC	Postexercise insulin sensitivity
Basal fasting glucose	**$r = -0.68$**	**$r = -0.61$**	**$r = -0.74$**	**$r = -0.58$**	**$r = -0.68$**
	$p < 0.01$	**$p < 0.01$**	**$p < 0.01$**	**$p = 0.01$**	**$p < 0.01$**
Basal fasting insulin	**$r = -0.46$**	**$r = -0.53$**	$r = -0.42$	$r = -0.44$	**$r = -0.78$**
	$p = 0.05$	**$p = 0.02$**	$p = 0.08$	$p = 0.07$	**$p < 0.01$**
Basal HOMA2-IR	$r = -0.45$	**$r = -0.54$**	$r = -0.42$	$r = -0.46$	**$r = -0.77$**
	$p = 0.06$	**$p = 0.02$**	$p = 0.09$	$p = 0.06$	**$p < 0.01$**
Basal fasting IL-6	$r = 0.40$	$r = 0.12$	$r = 0.32$	$r = 0.14$	$r = 0.39$
	$p = 0.13$	$p = 0.65$	$p = 0.22$	$p = 0.61$	$p = 0.14$
IL-6 after postexercise insulin stimulation	$r = -0.44$	**$r = -0.69$**	**$r = -0.58$**	**$r = -0.73$**	$r = -0.42$
	$p = 0.09$	**$p < 0.01$**	**$p = 0.02$**	**$p < 0.01$**	$p = 0.11$
Postexercise insulin sensitivity	**$r = 0.66$**	**$r = 0.72$**	**$r = 0.68$**	**$r = 0.60$**	
	$p < 0.01$	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	
p-mTOR^{Ser2481}	**$r = 0.76$**	**$r = 0.61$**	**$r = 0.71$**	**$r = 0.60$**	**$r = 0.54$**
	$p < 0.01$	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	**$p = 0.02$**
p-Akt^{Ser473}	**$r = 0.58$**	**$r = 0.71$**	**$r = 0.61$**	**$r = 0.62$**	**$r = 0.75$**
	$p = 0.01$	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**
p-AS160^{Thr642}	**$r = 0.56$**	**$r = 0.64$**	**$r = 0.57$**	**$r = 0.69$**	**$r = 0.56$**
	$p = 0.02$	**$p < 0.01$**	**$p = 0.01$**	**$p < 0.01$**	**$p = 0.02$**
p-AS160^{Ser318}	$r = 0.42$	**$r = 0.53$**	$r = 0.45$	**$r = 0.48$**	**$r = 0.50$**
	$p = 0.08$	**$p = 0.02$**	$p = 0.06$	**$p < 0.05$**	**$p = 0.04$**

Spearman rank correlation analysis was performed on combined data (prednisolone and placebo trials). The percent change in phosphorylation from baseline until after the clamp was used for correlation analysis. $N = 16$ for IL-6 correlations; $N = 18$ for all other correlations.

Bold text = significant correlation ($p < 0.05$).

tOC = Total osteocalcin; ucOC = undercarboxylated osteocalcin; HOMA2-IR = the homeostatic model assessment of insulin resistance version 2.

Serum IL-6 significantly increased after exercise and remained elevated after the insulin clamp with both GC and placebo (Fig. 2*E*). Compared with placebo, GC decreased serum IL-6 at baseline and immediately after exercise, but not 3 hours postexercise or after the insulin clamp. After the insulin clamp, higher tOC and ucOC correlated with lower serum IL-6 (Table 3).

Glucocorticoids decrease muscle insulin protein signaling

GC decreased GPRC6A protein abundance at all time points (Fig. 3*A*), and attenuated downstream signaling targets including insulin-stimulated p-Akt^{Ser473} and p-mTOR^{Ser2481}, but not p-ERK or p-AMPK, and increased baseline p-Akt^{Ser473} (Fig. 3*B–E*). GC increased baseline p-IRS1^{Ser307}, p-AS160^{Thr642}, and p-AS160^{Ser318}, which attenuated the percent increase in phosphorylation induced by exercise and the insulin clamp (Fig. 4*A–D*). Impairment of p-AS160, p-mTOR, and p-Akt signaling correlated with decreased insulin sensitivity and suppressed tOC and ucOC (Table 3). Postexercise insulin-stimulated p-AS160$^{Ser318,\ Thr642}$, p-mTOR^{Ser2481}, and p-Akt^{Ser473} were all significantly correlated (Supplementary Table 1). Serum IL-6 after the insulin clamp negatively correlated with insulin-stimulated p-AS160^{Thr642} ($r = -0.57$, $p = 0.02$).

Representative blots for Western blot analysis are provided in Supplementary Fig. 2 and graphical representations of linear regression analyses are provided in Supplementary Figs. 3 and 4.

Discussion

We report that in healthy young men, a single dose of GC induced basal insulin resistance, evident by increased fasting glucose, fasting insulin, and HOMA2-IR, and also decreased postexercise insulin sensitivity: features that were all associated with suppressed ucOC and tOC. Suppressed ucOC and insulin sensitivity were associated with decreased skeletal muscle mTOR–Akt–AS160 axis protein signaling, highlighting a novel signaling pathway for GC-induced insulin resistance in humans.

Osteocalcin, bone remodeling markers, and insulin resistance/sensitivity at rest and following exercise

Chronic GC treatment impairs insulin sensitivity in the liver, and in muscle and adipose tissues. However, short-term GC treatment decreases insulin sensitivity largely by reducing glucose disposal by muscle.[29,30] This loss of insulin sensitivity appears to be driven mainly by suppressed tOC and ucOC synthesis by osteoblasts, at least in murine models.[12] Transgenic mice overexpressing the glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2, which blocks glucocorticoid signaling in osteoblasts, maintain osteocalcin and ucOC levels and are protected from GC-induced insulin resistance.[12]

We report that suppression of ucOC and tOC with GC was associated with the development of insulin resistance in healthy young men. Furthermore, the suppression of insulin sensitivity following exercise with GC was associated with lower ucOC and tOC. Previous work in animals supports ucOC as a positive regulator of glycemic control.[1,3,4,31] In humans, we reported that the increase in ucOC following acute exercise correlates with improved glycemic control.[7,8] However, previous studies have only investigated whether the exercise-induced increase in osteocalcin secretion correlates with postexercise insulin sensitivity. We extend previous findings by providing evidence

that suppressing osteocalcin in humans before and after exercise is associated with decreased insulin sensitivity, supporting ucOC, and perhaps tOC, as a regulator of basal and postexercise human glycemic control.

GC treatment and exercise affect other markers of bone remodeling, including P1NP and β-CTx.[14,16] We report that acute high-intensity cycling exercise in men decreases serum β-CTx for up to 3 hours after exercise and remains suppressed during 2 hours of glucose and insulin infusion. P1NP was largely unaffected by acute exercise, but decreased during insulin and glucose infusion. Furthermore, GC significantly decreased P1NP at all time points and attenuated the suppression of serum P1NP and β-CTx during insulin and glucose infusion. In contrast to osteocalcin (tOC and ucOC), there were no correlations between serum P1NP, β-CTx, and measures of glycemic control. These findings support that tOC and ucOC, at least in humans, likely play a larger role then P1NP or β-CTx in whole-body glucose metabolism.

Research in rodents has identified a potential feed-forward loop that exists between exercise-induced IL-6 and osteocalcin that favors the many adaptations of regular exercise including whole-body energy metabolism.[16] However, despite a reduction in IL-6 with GC at baseline and immediately after exercise, we report similar serum IL-6 levels 3 hours after exercise and after insulin stimulation with the clamp. Furthermore, IL-6 after the clamp was negatively correlated with tOC and ucOC, which, if anything, suggests IL-6 as a potential negative regulator in humans of circulating osteocalcin during hyperinsulinemia clamp conditions. Further research in humans is required to confirm this.

The suppression of bone remodeling markers, including tOC, ucOC, P1NP, and β-CTx, has previously been reported following nutrient intake such as an oral glucose tolerance test or a mixed meal or glucose infusion during an euglycemic-hyperinsulinemic clamp.[32–35] In support of previous findings, we report that tOC, ucOC, P1NP, and β-CTx are suppressed in healthy men after insulin and glucose infusion, supporting the bidirectional feed-forward loop between osteocalcin and glucose. Interestingly, the suppression of bone remodeling markers following insulin/glucose infusion occurred to a lesser extent with GC ingestion, possibly because of already suppressed levels. Further research is required to explore the complex interaction between GC, feeding, and bone remodeling markers.

Glucocorticoid suppression of osteocalcin-associated skeletal muscle protein signaling

GPRC6A is an amino acid-sensing G protein-coupled receptor expressed in tissues responsive to ucOC including skeletal muscle.[16,19,36] Mice lacking GPRC6A develop insulin resistance, high circulating insulin, and fat accumulation.[1,37–39] In addition, hind-limb immobilization in rats suppresses ucOC and GPRC6A expression.[40] We report decreased GPRC6A protein expression in human skeletal muscle with GC; however, this suppression was not correlated with ucOC or tOC. It is possible that GPRC6A protein expression in human skeletal muscle does not adequately reflect ucOC-mediated activation of the receptor. Although GPRC6A appears to be the predominant osteocalcin receptor in skeletal muscle of mice,[16] whether this is true in humans is unclear. Further mechanistic studies exploring ucOC and skeletal muscle GPRC6A signaling in humans are required.

Intermittent ucOC treatment restores insulin sensitivity in high-fat-diet-fed mice likely through restoration of the IRS, mTOR, and Akt signaling cascade.[18,41] Furthermore, in mice,

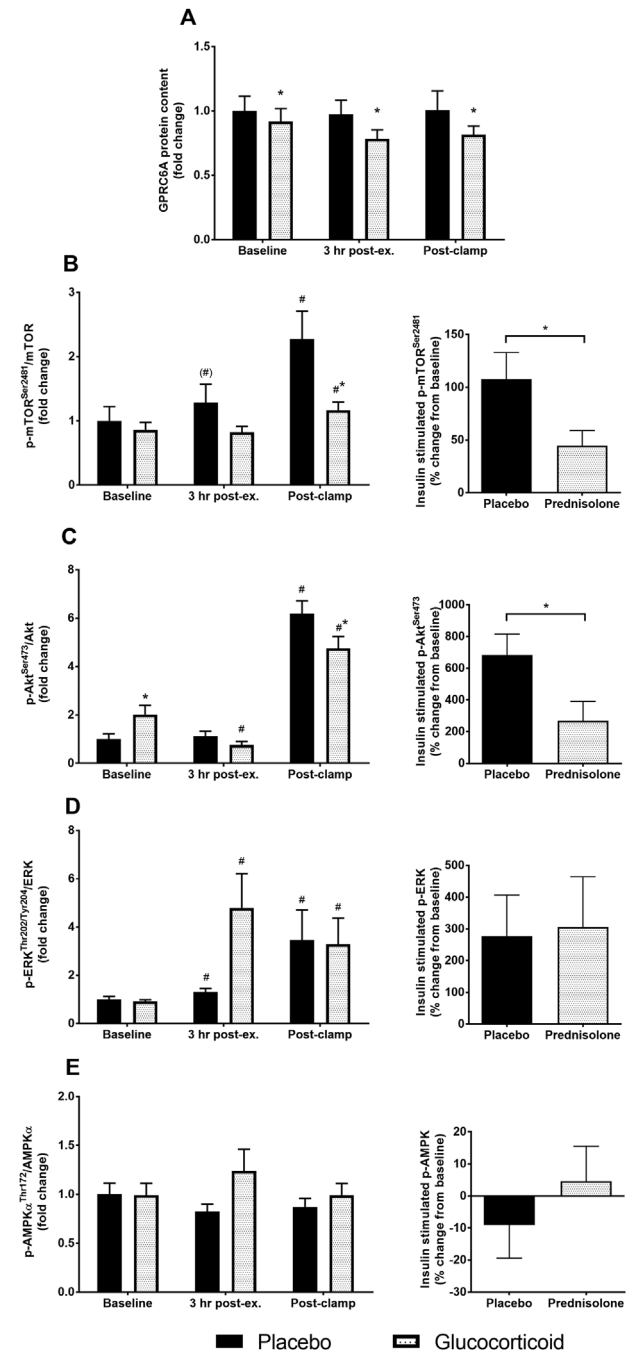

Fig. 3. The effect of glucocorticoid (prednisolone) on baseline, postexercise, and postclamp (after insulin and glucose infusion) skeletal muscle protein signaling. (*A*) GPRC6A protein abundance, (*B*) p-mTOR^{Ser2481}, (*C*) p-Akt^{Ser473}, (*D*) p-Erk$^{Thr202/Tyr204}$, and (*E*) p-AMPK^{Thr172}. The postclamp percent increase was calculated by expressing the postclamp value of each participant relative to their baseline sample. Comparisons of multiple means were examined using a 2-factor (capsule ingestion × time point) repeated measures analysis of variance using Fisher's least significant difference where appropriate. Two-tailed paired *t* tests were conducted on the percent change in insulin-stimulated phosphorylation between placebo and glucocorticoid. * = Significant difference ($p < 0.05$) compared with placebo trial; # = significant difference ($p < 0.05$) compared with baseline. Symbols in parenthesis are $p < 0.1$, $N = 9$. All data are presented as mean ± SEM.

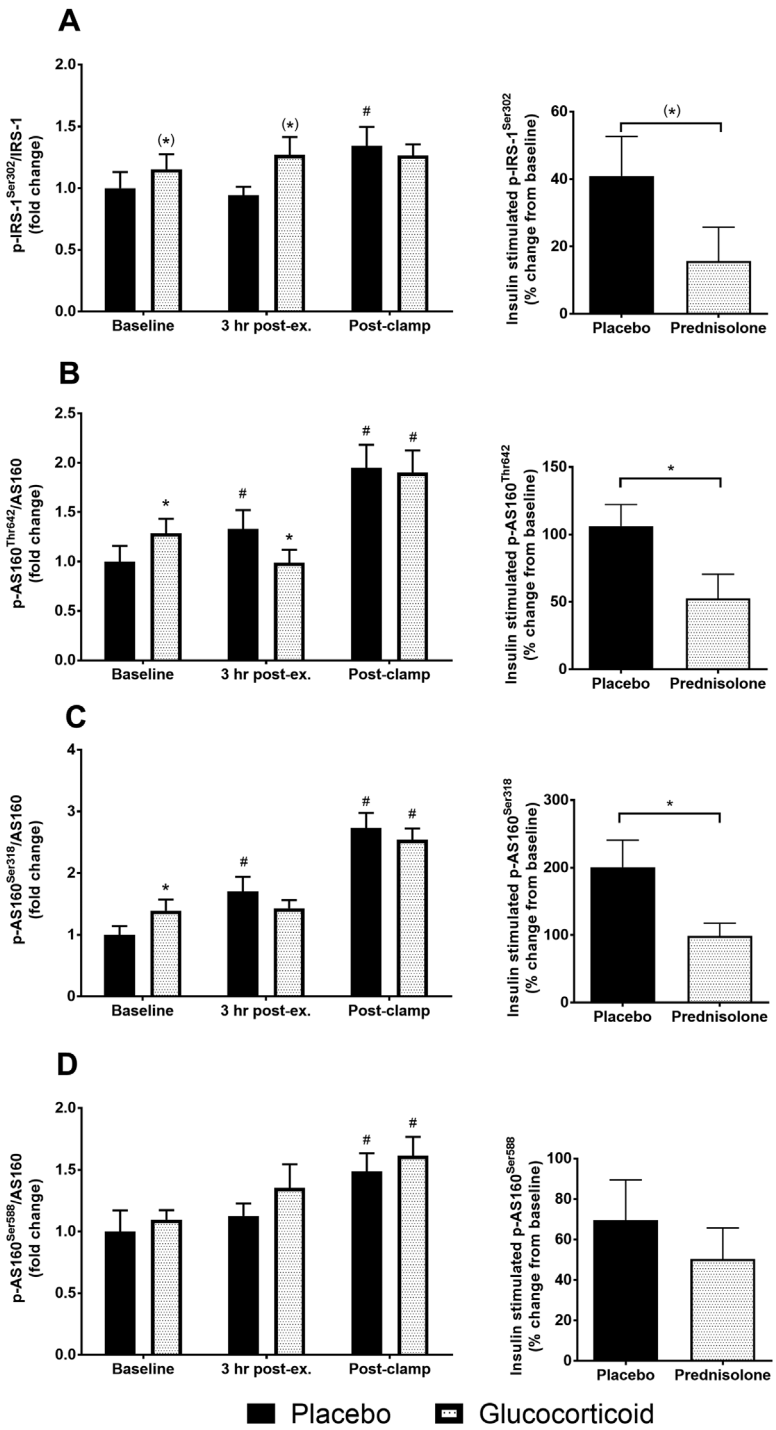

Fig. 4. The effect of glucocorticoid (prednisolone) on baseline, postexercise, and postclamp (after insulin and glucose infusion) skeletal muscle protein signaling. (*A*) p-IRS-1^{Ser302}, (*B*) p-AS160^{Thr642}, (*C*) p-AS160^{Ser318}, and (*D*) p-AS160^{Ser588}. The postclamp percent increase was calculated by expressing the postclamp value of each participant relative to their baseline sample. Comparisons of multiple means were examined using a 2-factor (capsule ingestion $\times$ time point) repeated measures analysis of variance using Fisher's least significant difference where appropriate. Two-tailed paired *t* tests were conducted on the percent change in insulin-stimulated phosphorylation between placebo and glucocorticoid. * = Significant difference ($p < 0.05$) compared with placebo trial; # = significant difference ($p < 0.05$) compared with baseline. Symbols in parenthesis are $p < 0.1$, $N = 9$. All data are presented as mean $\pm$ SEM.

ucOC increases insulin-stimulated AS160 phosphorylation and glucose uptake following ex vivo muscle contraction.[4] We report that activation of insulin protein signaling is associated with ucOC in humans. We observed impairment of p-mTOR^{Ser2481}, p-Akt^{Ser473}, and p-AS160$^{Thr642,\ Ser318}$ with GC following exercise and insulin stimulation, which correlated with suppressed tOC and ucOC and insulin sensitivity. Interestingly, GC increased baseline p-Akt^{Ser473}, p-IRS1^{Ser307}, and p-AS160$^{Thr642,\ Ser318}$, possibly compensating for increased insulin resistance and insulin secretion at baseline. Furthermore, phosphorylation of ERK was not affected by GC ingestion, supporting previous work suggesting a lesser role of ERK in GC-induced insulin resistance.[42] Our findings also suggest that AMPK, which was not altered by insulin stimulation or GC, may play a lesser role in ucOC-mediated glucose uptake in humans; we have previously reported a similar finding in mice.[31] However, it is also possible that the AMPKα (Thr172) phosphorylation site detected by our antibody may not adequately reflect AMPK activity.[43] As such, future studies would benefit by directly measuring AMPK activity.

Limitations

Some of the effects of GC on insulin sensitivity may be because of the effects of GC on the liver, adipose tissue, or other signaling pathways in muscle. Nevertheless, short-term (5 days) GC therapy has minimal effects on muscle protein synthesis and breakdown, mitochondrial function, strength, and resting energy expenditure in men, despite increased insulin resistance.[44] Similarly, perturbed whole-body energy metabolism in rodents precedes changes to canonical GC receptor signaling in skeletal muscle and liver,[12] whereas the effect of GC on osteoblast function and osteocalcin synthesis in humans occurs within hours.[14,15] A limitation of the current study is the use of the HOMA2-IR, which is not the gold standard for measuring glucose and insulin dynamics including hepatic insulin sensitivity. However, the primary aim of this study was to investigate the effects of GC ingestion on postexercise insulin sensitivity—for which we used the gold standard euglycemic–hyperinsulinemic clamp. These findings are limited to young healthy adult men and may not be generalizable to other populations.

Conclusion

We provide evidence that GC-induced basal and postexercise insulin resistance in humans is associated with the suppression of circulating ucOC and ucOC-linked protein signaling in skeletal muscle. Whether ucOC treatment can offset GC-induced insulin resistance in humans requires further investigation.

Disclosure

The authors report no conflicts of interest in this work.

Acknowledgments

This study was funded by Victoria University. A/Prof Levinger was supported by a Future Leader Fellowship (ID: 100040) from the National Heart Foundation of Australia. Dr Parker was supported by an Alfred Deakin Postdoctoral Fellowship from Deakin University. Dr Brennan-Speranza was supported by an NHMRC Early Career Fellowship. A/Prof Nigel Stepto was supported by the Australian Governments Collaborative Research Network.

Thank you to the participants involved in the research and Alex Tacey and Emma McLennan for assisting with data collection.

Authors' roles: Study design: All authors. Study conduct: LP, AG, NS, and IL. Data collection: LP, AG, NS, and IL. Data analysis: LP and EB. Data interpretation: All authors. Drafting manuscript: LP and IL. Revising manuscript content: All authors. Approving final version of manuscript: All authors. LP and IL take responsibility for the integrity of the data analysis.

References

1. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. Aug 10 2007;130(3):456–69. Epub 2007 Aug 19.
2. Levinger I, Brennan-Speranza TC, Zulli A, et al. Multifaceted interaction of bone, muscle, lifestyle interventions and metabolic and cardiovascular disease: role of osteocalcin. Osteoporos Int. Aug 2017;28(8):2265–73. Epub 2017 Mar 16.
3. Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc Natl Acad Sci U S A. Apr 1 2008;105(13):5266–70. Epub 2008 Mar 26.
4. Levinger I, Lin X, Zhang X, Brennan-Speranza TC, Volpato B, Hayes A, et al. The effects of muscle contraction and recombinant osteocalcin on insulin sensitivity ex vivo. Osteoporos Int. 2016;27(2):653–63. Epub 2015 Aug 12.
5. Kanazawa I, Yamaguchi T, Yamauchi M, et al. Serum undercarboxylated osteocalcin was inversely associated with plasma glucose level and fat mass in type 2 diabetes mellitus. Osteoporos Int. 2011;22(1):187–94. Epub 2010 Feb 19.
6. Liu DM, Guo XZ, Tong HJ, et al. Association between osteocalcin and glucose metabolism: a meta-analysis. Osteoporos Int. 2015;26(12):2823–33. Epub 2015 Jun 20.
7. Levinger I, Jerums G, Stepto NK, et al. The effect of acute exercise on undercarboxylated osteocalcin and insulin sensitivity in obese men. J Bone Miner Res. 2014;29(12):2571–6. Epub 2014 May 28.
8. Levinger I, Zebaze R, Jerums G, Hare DL, Selig S, Seeman E. The effect of acute exercise on undercarboxylated osteocalcin in obese men. Osteoporos Int. 2011;22(5):1621–6. Epub 2010 Aug 25.
9. Kim YS, Nam JS, Yeo DW, Kim KR, Suh SH, Ahn CW. The effects of aerobic exercise training on serum osteocalcin, adipocytokines and insulin resistance on obese young males. Clin Endocrinol (Oxf). 2015;82(5):686–94. Epub 2014 Sep 2.
10. Brennan-Speranza TC, Conigrave AD. Osteocalcin: an osteoblast-derived polypeptide hormone that modulates whole body energy metabolism. Calcif Tissue Int. 2015;96(1):1–10. Epub 2014 Nov 25.
11. Parker L, Shaw CS, Stepto NK, Levinger I. Exercise and glycemic control: focus on redox homeostasis and redox-sensitive protein signaling. Front Endocrinol (Lausanne). 2017;8(87):87. Epub 2017 May 23.
12. Brennan-Speranza TC, Henneicke H, Gasparini SJ, et al. Osteoblasts mediate the adverse effects of glucocorticoids on fuel metabolism. J Clin Invest. 2012;122(11):4172–89. Epub 2012 Oct 25.
13. Henneicke H, Gasparini SJ, Brennan-Speranza TC, Zhou H, Seibel MJ. Glucocorticoids and bone: local effects and systemic implications. Trends Endocrinol Metab. 2014;25(4):197–211. Epub 2014 Jan 15.
14. Fleishaker DL, Mukherjee A, Whaley FS, Daniel S, Zeiher BG. Safety and pharmacodynamic dose response of short-term prednisone in healthy adult subjects: a dose ranging, randomized, placebo-controlled, crossover study. BMC Musculoskelet Disord. 2016;17:293. Epub 2016 Jul 18.
15. Nielsen HK, Charles P, Mosekilde L. The effect of single oral doses of prednisone on the circadian rhythm of serum osteocalcin in normal subjects. J Clin Endocrinol Metab. 1988;67(5):1025–30. Epub 1988 Nov 1.

16. Mera P, Laue K, Ferron M, et al. Osteocalcin signaling in myofibers is necessary and sufficient for optimum adaptation to exercise. Cell Metab. 2016;23(6):1078–92. Epub 2016 Jun 16.
17. Mera P, Laue K, Wei J, Berger JM, Karsenty G. Osteocalcin is necessary and sufficient to maintain muscle mass in older mice. Mol Metab. 2016;5(10):1042–7. Epub 2016 Oct 1.
18. Zhou B, Li H, Liu J, et al. Autophagic dysfunction is improved by intermittent administration of osteocalcin in obese mice. Int J Obes (Lond). 2016;40(5):833–43. Epub 2016 Jan 8.
19. Pi M, Nishimoto SK, Quarles LD. GPRC6A: Jack of all metabolism (or master of none). Mol Metab. 2017;6(2):185–93. Epub 2017 Feb 10.
20. Levinger I, Brennan-Speranza TC, Stepto NK, et al. A single dose of prednisolone as a modulator of undercarboxylated osteocalcin and insulin sensitivity post-exercise in healthy young men: a study protocol. JMIR Res Protoc. 2016;5(2):e78. Epub 2016 Jun 5.
21. Heuck C, Wolthers OD. A placebo-controlled study of three osteocalcin assays for assessment of prednisolone-induced suppression of bone turnover. J Endocrinol. 1998;159(1):127–31. Epub 1998 Oct 31.
22. Frosig C, Richter EA. Improved insulin sensitivity after exercise: focus on insulin signaling. Obesity (Silver Spring). 2009;17(Suppl 3): S15–20. Epub 2009 Nov 21.
23. Howlett KF, Mathews A, Garnham A, Sakamoto K. The effect of exercise and insulin on AS160 phosphorylation and 14-3-3 binding capacity in human skeletal muscle. Am J Physiol Endocrinol Metab. 2008;294(2):E401–7. Epub 2007 Nov 29.
24. Evans WJ, Phinney SD, Young VR. Suction applied to a muscle biopsy maximizes sample size. Med Sci Sports Exerc. 1982;14(1):101–2. Epub 1982 Jan 1.
25. Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. J Clin Endocrinol Metab. 1998;83(9): 3258–66. Epub 1998 Sep 24.
26. Murphy RM, Lamb GD. Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. J Physiol. 2013;591(23):5823–31. Epub 2013 Oct 16.
27. Mollica JP, Oakhill JS, Lamb GD, Murphy RM. Are genuine changes in protein expression being overlooked? Reassessing Western blotting. Anal Biochem. 2009;386(2):270–5. Epub 2009 Jan 24.
28. Parker L, Stepto NK, Shaw CS, et al. Acute high-intensity interval exercise-induced redox signaling is associated with enhanced insulin sensitivity in obese middle-aged men. Front Physiol. 2016; 7:411. Epub 2016 Oct 4.
29. Larsson H, Ahren B. Insulin resistant subjects lack islet adaptation to short-term dexamethasone-induced reduction in insulin sensitivity. Diabetologia. 1999;42(8):936–43. Epub 1999 Sep 24.
30. Wajngot A, Giacca A, Grill V, Vranic M, Efendic S. The diabetogenic effects of glucocorticoids are more pronounced in low-insulin than in high-insulin responders. Proc Natl Acad Sci U S A. 1992;89(13): 6035–9.
31. Lin X, Parker L, McLennan E, et al. Recombinant uncarboxylated osteocalcin per se enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles. Front Endocrinol (Lausanne). 2017;8(330):330. Epub 2017 Dec 6.
32. Levinger I, Brennan-Speranza TC, Jerums G, et al. The effect of hyperinsulinaemic-euglycaemic clamp and exercise on bone remodeling markers in obese men. Bonekey Rep. 2015;4:731. Epub 2015 Sep 4.
33. Paldanius PM, Ivaska KK, Hovi P, et al. The effect of oral glucose tolerance test on serum osteocalcin and bone turnover markers in young adults. Calcif Tissue Int. 2012;90(2):90–5. Epub 2011 Dec 8.
34. Lopes LS, Schwartz RP, Ferraz-de-Souza B, da Silva ME, Correa PH, Nery M. The role of enteric hormone GLP-2 in the response of bone markers to a mixed meal in postmenopausal women with type 2 diabetes mellitus. Diabetol Metab Syndr. 2015;7:13. Epub 2015 Mar 10.
35. Levinger I, Seeman E, Jerums G, et al. Glucose-loading reduces bone remodeling in women and osteoblast function in vitro. Physiol Rep. 2016;4(3):e12700. Epub 2016 Feb 6.
36. Pi M, Quarles LD. Multiligand specificity and wide tissue expression of GPRC6A reveals new endocrine networks. Endocrinology. 2012;153(5):2062–9. Epub 2012 Mar 1.
37. Clemmensen C, Smajilovic S, Madsen AN, Klein AB, Holst B, Brauner-Osborne H. Increased susceptibility to diet-induced obesity in GPRC6A receptor knockout mice. J Endocrinol. 2013;217(2):151–60. Epub 2013 Feb 23.
38. Pi M, Chen L, Huang MZ, et al. GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. PLoS ONE. 2008;3(12):e3858. Epub 2008 Dec 4.
39. Oury F, Sumara G, Sumara O, et al. Endocrine regulation of male fertility by the skeleton. Cell. 2011;144(5):796–809. Epub 2011 Feb 22.
40. Lin X, Hanson E, Betik AC, Brennan-Speranza TC, Hayes A, Levinger I. Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. J Bone Miner Res. 2016;31(11):1967–78. Epub 2016 Jun 14.
41. Ferron M, McKee MD, Levine RL, Ducy P, Karsenty G. Intermittent injections of osteocalcin improve glucose metabolism and prevent type 2 diabetes in mice. Bone. 2012;50(2):568–75. Epub 2011 May 10.
42. Bazuine M, Carlotti F, Tafrechi RS, Hoeben RC, Maassen JA. Mitogen-activated protein kinase (MAPK) phosphatase-1 and -4 attenuate p38 MAPK during dexamethasone-induced insulin resistance in 3T3-L1 adipocytes. Mol Endocrinol. 2004;18(7):1697–707. Epub 2004 Jun 9.
43. Kjobsted R, Munk-Hansen N, Birk JB, et al. Enhanced muscle insulin sensitivity after contraction/exercise is mediated by AMPK. Diabetes. 2017;66(3):598–612.
44. Short KR, Nygren J, Bigelow ML, Nair KS. Effect of short-term prednisone use on blood flow, muscle protein metabolism, and function. J Clin Endocrinol Metab. 2004;89(12):6198–207. Epub 2004 Dec 8.