The Effect of Acute Exercise on Undercarboxylated Osteocalcin and Insulin Sensitivity in Obese Men[†]

Running title: Exercise, undercarboxylated osteocalcin and insulin sensitivity in obese men

Itamar Levinger^{1,2*}, George Jerums³, Nigel K Stepto¹, Lewan Parker¹, Fabio R Serpiello¹, Glenn K McConell^{1,2}, Mitchell Anderson¹, David L. Hare⁴, Elizabeth Byrnes⁵, Peter R Ebeling², Ego Seeman³

¹ Institute of Sport, Exercise and Active Living (ISEAL), College of Sport and Exercise Science, Victoria University, Melbourne, Australia.

² Australian Institute of Musculoskeletal Science, NorthWest Academic Centre, The University of Melbourne, Western Health.

³ University of Melbourne and the Department of Endocrinology, Austin Health, Melbourne, Australia

⁴ University of Melbourne and the Department of Cardiology, Austin Health, Melbourne Australia.

⁵ PathWest QEII Medical Centre, Perth, Australia

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* Address for correspondence:

Dr Itamar Levinger

Institute of Sport, Exercise and Active Living (ISEAL),

Victoria University

PO Box 14428, Melbourne, VIC 8001, Australia.

Tel: (61-3) 9919 5343, Fax: (61-3) 9919 5532, E-mail: itamar.levinger@vu.edu.au

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Abstract

Acute exercise improves insulin sensitivity for hours after the exercise is ceased. The skeleton contributes to glucose metabolism and insulin sensitivity via osteocalcin (OC) in its undercarboxylated (ucOC) form in mice. We tested the hypothesis that insulin sensitivity over the hours following exercise is associated with circulating levels of ucOC. Eleven middle-aged (58.1 ± 2.2 year mean $\pm$ SEM), obese ($\text{BMI} = 33.1 \pm 1.4 \text{ kg} \cdot \text{m}^{-2}$) non-diabetic men completed a euglycaemic-hyperinsulinaemic clamp at rest (Rest-Control) and at 60 min post-exercise (4×4 min of cycling at 95% of HR_{peak}). Insulin sensitivity was determined by glucose infusion rate relative to body mass (GIR , $\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) as well as GIR per unit of insulin (M-value). Blood samples and 5 muscle biopsies were obtained; two at the Resting-control session, one before and one after clamping, and three in the Exercise session, at rest, 60min post-exercise and after the clamp. Exercise increased serum ucOC ($6.4 \pm 2.1\%$, $p = 0.013$) but not total OC ($p > 0.05$). Blood glucose was $\sim 6\%$ lower and insulin sensitivity was $\sim 35\%$ higher post-exercise compared with control (both $p < 0.05$). Phosphorylated (P)-AKT (Akt thymoma) was higher after exercise and insulin compared to exercise alone (no insulin) and insulin alone (no exercise, all $p < 0.05$). In a multiple-linear regression including BMI, age and aerobic fitness, ucOC was associated with whole body insulin sensitivity at rest ($\beta = 0.59$, $p = 0.023$) and post-exercise ($\beta = 0.66$, $p = 0.005$). Insulin sensitivity, following acute exercise, is associated with circulating levels of ucOC in obese men. Whether ucOC has a direct effect on skeletal muscle insulin sensitivity after exercise is yet to be determined.

Keywords: bone metabolism; exercise; glycaemic control; obesity; undercarboxylated osteocalcin

Introduction

Exercise is important for the prevention and management of type 2 diabetes (T2DM). Even a single bout of exercise increases insulin sensitivity for hours after exercise is ceased ^(1, 2). This increase in sensitivity occurs in skeletal muscle, a major site for glucose disposal. Glucose disposal rate increases during and after exercise, even in those with elevated body mass and poorer glycaemic control ⁽³⁾. However, the factors responsible for this increase in insulin sensitivity post-exercise are not completely understood ⁽⁴⁾.

Recent work suggests that the skeleton is an endocrine organ that participates in glucose homeostasis ^(5, 6). Osteocalcin (OC) is an osteoblast-specific product that is secreted into the bone extracellular matrix and the general circulation and reflects bone remodeling ⁽⁷⁾. In murine models, the undercarboxylated form of OC (ucOC) stimulates β -cell proliferation and insulin secretion and ucOC-deficient mice are obese, glucose intolerant and have features of T2DM ⁽⁶⁾. In humans, ucOC, and OC, are also correlated with insulin sensitivity, fasting glucose, fat mass and muscle strength ⁽⁸⁻¹¹⁾. Moreover, serum ucOC is associated with improved glucose tolerance in middle-aged men, an effect that may be related to enhanced β -cell function ⁽¹²⁾. In addition, acute exercise increases ucOC, and this increase in ucOC is related to a reduction in serum glucose levels in obese men ⁽¹³⁾. It is unclear whether increases in ucOC, are related with improvement in whole body insulin sensitivity post-exercise, nor whether serum ucOC levels predict changes in skeletal muscle insulin signalling including AKT and AS160. Akt and its 160 kDa substrate (AS160) are downstream proteins in the phosphatidylinositol 3-kinase (PI3-K) pathway and may be a convergence point between the pathways regulating insulin- and contraction-stimulated GLUT-4 translocation ^(14, 15).

The aim of this study was tested the hypothesis that in obese men, insulin sensitivity following exercise is related to the circulating levels of ucOC.

Materials and Methods

Participants: Eleven middle-aged (58.1 ± 2.2 year mean $\pm$ SEM, range 40-70 years), obese ($BMI = 33.1 \pm 1.4 \text{ kg} \cdot \text{m}^{-2}$) non-diabetic men participated in the study (Table 1). Power and precision software was used to calculate the needed sample size (power of 80%, $\alpha = 0.05$) to detect changes ($>6\%$) in serum ucOC following acute aerobic exercise ⁽¹³⁾. We excluded men with bone disease, men taking anti-hyperglycaemic medications or medications known to affect bone metabolism, insulin secretion or insulin sensitivity, musculoskeletal or other

conditions that prevent daily activity, men with symptomatic or uncontrolled metabolic or cardiovascular disease, or receiving warfarin or vitamin K supplementation.

Each participant was given written and verbal explanations about the study before signing an informed consent form. The study protocol was approved by the Human Research Ethics Committee, Victoria University.

Study protocol: Participants underwent anthropometric measurements, assessment of their aerobic power ($\text{VO}_{2\text{peak}}$) and a fasting blood test. Participants also completed two sessions of 2h euglycaemic-hyperinsulinaemic clamp (insulin clamp), once at rest (Rest-Control) and once commenced 60 min after acute bout of exercise (post-exercise). During the two insulin clamp sessions five muscle biopsies were obtained from each participant (Figure 1).

A blood sample was collected following overnight fast. Blood was analysed at Austin Health (Melbourne, Australia) pathology using the standard hospital assay protocols for triglyceride, high-density lipoprotein (HDL), glucose, HbA1c and insulin.

Blood pressure was measured using a standard mercury sphygmomanometer after the participant had rested in a seated position for at least 15 min.

Weight was measured using a scale (TANITA, Tanita Corporation, Tokyo, Japan).

Aerobic power ($\text{VO}_{2\text{peak}}$) was assessed during a sign and symptom-limited graded exercise test as described ⁽¹⁶⁾.

VO_2 for each 15 sec interval was measured by gas analysis (Medgraphics, Cardio2 and CPX/D System with Breezeex Software, 142090-001, Revia, MN, USA) that was calibrated before each test.

Experimental sessions: a diagram of the two experimental sessions is illustrated in Figure 1. Participants attended our laboratory twice for the experimental trials, at 8am after an overnight fast. In the day prior to the experiment volunteers were asked to consume around 300g of carbohydrate, to avoid glycogen depletion. The two experimental trials (Control-Rest or exercise) were conducted 3-5 weeks apart.

Control-Rest session, to determine the basal (rest) insulin sensitivity as well as resting levels of OC and uOC.

Euglycemic-hyperinsulinemic clamp (insulin clamp) was performed at rest as was reported previously ^(17, 18).

Briefly, the clamps were performed after an overnight fast. Venous blood samples, heated arm vein, were collected prior and during each session. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused at $40\text{mU}\cdot\text{m}^{-2}$ per minute for 120 minutes generating an elevated, stable insulin concentration in the last 30 minutes

of both sessions (Rest-control: 70.5 ± 7.4 mIU·L⁻¹ and Exercise 67.1 ± 6.2 mIU·L⁻¹, $p < 0.001$ compared to baseline levels) confirming a hyperinsulinemic state ($> 24.9 \mu\text{U} \cdot \text{mL}^{-1}$). Insulin sensitivity was assessed by the glucose infusion rate (GIR, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) during the last 30 minutes of the insulin-stimulated period and the GIR per unit of insulin (M-Value)⁽¹⁹⁾. During both hyperinsulinemic-euglycemic clamps sessions, exogenous glucose was variably infused to achieve the target blood glucose of $\sim 5 \text{ mmol/L}$ for the duration of the clamp, using variable infusion. Glucose was assessed every 5 min during the clamp (YSI2300 STAT Plus™ Glucose & Lactate Analyser, Australia). Two muscle biopsies were taken during this session, one before and one after the insulin clamp.

In the second session, an insulin clamp following an acute high intensity bout of exercise was performed. Participants were supine and a resting blood sample and resting muscle biopsy were taken. After the initial blood sampling and muscle biopsy participants performed 30 minutes of high intensity interval exercise that included one warm-up set of 4 min of exercise at approximately 50-60% of HR_{peak} followed by 4 sets (bouts) of 4 min each at 90-95% of HR_{peak} . The high intensity intervals were separated by 2 min of 'active' recovery (cycling at a lower intensity (50-60% of peak). Blood samples were obtained immediately post-exercise and at 30 and 60 min post-exercise to identify the peak change in OC and ucOC, glucose and insulin. The insulin clamp commenced 60 min post-exercise and it was performed as described in Session 1. Additional muscle biopsies were taken before and after the insulin clamp. Overall, 3 muscle biopsies were taken during session 2, at rest, 60 min post-exercise and post-insulin clamp.

Muscle biopsies. Muscle Samples were obtained from the vastus lateralis under local anaesthesia (Xylocaine 1%), utilizing the percutaneous needle biopsy technique with suction⁽²⁰⁾. The samples were immediately frozen in liquid nitrogen and were then stored at -80°C until analysis.

Serum osteocalcin and undercarboxylated osteocalcin: Total serum OC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics). This assay has a sensitivity of $0.5 \mu\text{g} \cdot \text{L}^{-1}$, with an intra-assay precision of 1.3%. 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 et al.⁽²¹⁾. The ucOC values on these samples of $6.0\text{-}35.6 \mu\text{g} \cdot \text{L}^{-1}$ and %unbound osteocalcin between 38.1-51.4% are in keeping with the original assay validation. The inter-assay CV for total OC is 8.3% and the inter-assay CV for ucOC is 5.7%.

Western blots: 3-4 mg of freeze dried skeletal muscle was homogenised with 150 μ L/mg of homogenising buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 20 mM Tris, 1 mM EDTA, 5 mM Na pyrophosphate, 10 mM NaF, 1 % Triton X-100, 10 % Glycerol, 200 μ M PMSF, 0.5 mM Na₄VO₃, 1 mM Benzamidine and 0.1 % protease inhibitor cocktail). Samples were rotated for 1 hour at 4 °C, centrifuged at 15000g (4 °C) for 15 minutes and the supernatant was collected. Protein content was determined using the Bradford Assay (Bio-Rad laboratories, Hercules, CA, USA) as per the manufacturer's instructions. All samples were diluted to a standard concentration of 2 μ g/ μ l using 4 x Laemmli buffer (containing 8 μ L β -mercaptoethanol per 100 μ L of Laemmli buffer) and homogenising buffer. Total protein (25 μ g) of each sample was loaded onto 7.5% Criterion™ TGX™ Gels and run for 45 minutes at 200 V. Samples were electroblotted (Criterion™ Blotter With Wire Electrodes) onto PVDF (0.45 mm pore) membrane in TOWBIN transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol, 0.1% SDS and pH 8.3) for 80 min at 100 V. Membranes were blocked with Tris-Buffered Saline-Tween (TBST: 0.1 M Tris Base, 1.5 M NaCl, 0.1% Tween-20) and 5% skim milk for 1 hour and then washed (5 x 8 minutes) with TBST. Membranes were incubated at 4 °C overnight with the following primary antibodies: Phospho-Akt (Ser473), Akt, AS160, Phospho-AS160 (Thr642) (Cell Signaling Technology, Danvers, MA) and α -Tubulin (Abcam, Cambridge, UK). After incubation membranes were washed with TBST, incubated for 1 hour at room temperature with appropriate dilutions of horseradish peroxidase conjugated secondary antibodies, re-washed and then exposed to Clarity™ Western ECL Substrate (Bio-Rad) for 1 minute. Membranes were scanned using a VersaDoc™ Imaging System (Bio-Rad) and densitometry analysed using Quantity One software (Bio-Rad). All densitometry values were then expressed relative to a pooled internal standard and a corresponding α -tubulin loading control from the equivalent sample lysate.

Statistical analyses

Changes from pre-to-post exercise within each trial and between trials were analysed by paired t-tests. The insulin signalling proteins AKT and AS160 (total and phosphorylated) were analysed using general linear model ANOVA with Bonferroni correction. Multi-linear regression model with ucOC, age, BMI and aerobic fitness were used to determine association with insulin sensitivity. All data are reported as mean $\pm$ standard error of mean (SEM) and all statistical analyses were conducted at the 95% level of significance ($p \leq 0.05$).

Results

The characteristics of participants are shown in Table 1. Blood glucose, insulin, tOC and ucOC were similar at baseline between the Rest-Control and Exercise sessions (all $p>0.05$, Table 2). At rest, in a multi-linear regression model that included ucOC, BMI, age and aerobic fitness has shown that both ucOC and BMI, but not age or aerobic fitness, were associated with resting insulin sensitivity (GIR, $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $\beta=0.59$, $p=0.023$, $\beta=-0.89$ $p=0.003$ respectively).

Effects of exercise

Exercise intensity during the session was $95.1\pm1.9\%$ of HR_{peak} . The mean rate of perceived exertion was 16.3 ± 0.4 (rating range 6-20). Exercise increased the circulating ucOC (10.6 ± 0.8 to 11.2 ng/ml $p<0.05$) and ucOC/OC ratio (58.9 ± 2.0 to $62.1\pm1.9\%$, $p=0.023$) but not tOC ($p>0.05$, Figure 2). Blood glucose decreased (from 5.3 ± 0.3 to 5.0 ± 0.3 , $p<0.05$, Figure 3) during the 60 min post-exercise, without change in serum insulin (13.8 ± 1.9 to 12.4 ± 2.4 mIU/L, $p=0.33$).

The glucose infusion rate (GIR, $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) needed to maintain blood glucose levels at $\sim 5\text{mmol}\cdot\text{L}^{-1}$ and the GIR per unit of circulating insulin (M-value), both measures of insulin sensitivity, were higher 3h post-exercise compared with the Rest-Control session ($p<0.05$, Figure 3).

There was no significant change in total AKT (Figure 4A) and total AS160 (Figure 4C) after any treatment (after a resting insulin clamp, after exercise alone and after exercise and insulin clamp $p>0.05$). At rest, p-AKT, the active form of AKT, did not significantly change with insulin (Rest-control session). However, after exercise and insulin (insulin clamp) p-AKT was significantly higher compared to exercise alone (no insulin, $p=0.004$, Figure 4B) and insulin alone (no exercise, $p=0.029$, Figure 4B). P-AS160 was higher following exercise and following exercise and insulin compared to baseline level (Figure 4D).

In a multi-linear regression model, ucOC level post-exercise was associated with insulin sensitivity (GIR and M-value) post-exercise ($\beta= 0.66$, $p=0.005$ and $\beta= 0.39$, $p=0.02$, respectively) as were BMI and age ($p<0.05$), but not aerobic fitness. Importantly, baseline ucOC level was also associated with post-exercise insulin sensitivity (GIR and M-value $\beta= 0.57$, $p=0.036$ and $\beta= 0.37$, $p=0.044$, respectively) as were age and BMI. The

percentage change in ucOC levels from pre-to-post exercise was not associated with the percentage change in whole body insulin sensitivity post-exercise. BMI, age and aerobic fitness were also not associated with the change in insulin sensitivity from pre-to-post exercise.

UcOC levels post-exercise was associated with the change in p-AKT after exercise and insulin ($\beta = 0.69$, $p=0.03$).

Discussion

We report that acute high intensity exercise increases ucOC levels and improves insulin sensitivity post-exercise. UcOC was associated with insulin sensitivity at rest and post-exercise. Insulin sensitivity, at rest and following acute exercise, is associated with circulating levels of ucOC in obese men.

In mice, the skeleton plays a role in glucose metabolism via a mechanism involving ucOC ⁽⁵⁾. Data from humans are supportive but are based on cross-sectional studies ^(8, 12). As acute exercise is known to increase whole body insulin sensitivity and glycaemic control, it can be used as a tool to examine the contribution of the skeleton to the insulin-sensitising effect of exercise. We have previously reported that acute aerobic exercise increased both tOC and ucOC in obese men, but only the increase in ucOC was related to an improvement in glycaemic control post-exercise ⁽¹³⁾. Insulin sensitivity and insulin signalling proteins were not measured in the previous study.

It is not clear why tOC did not change in the current study as previously we have reported that tOC increases with aerobic exercise ⁽¹³⁾. It is possible that the different result is related to the exercise intensity or duration. In the current study, participants exercised for 30 min at ~95% of HR_{peak}, compared with 45 min at 75% of HR_{peak} in the previous study. Other studies also reported no change in tOC following aerobic exercise ⁽²²⁻²⁵⁾. Nevertheless, insulin sensitivity improved in the absence of a change in tOC suggesting that it is the undercarboxylated form of OC, and not the carboxylated OC, that has a metabolic role in humans, as it does in mice.

There is increasing evidence that ucOC is related to glycaemic control in humans, however causality has not been determined. Although association does not imply causation, our results add new information about a possible connection between ucOC and insulin sensitivity in humans. Serum ucOC at baseline was associated with insulin sensitivity and p-AKT at rest and the change in insulin sensitivity post-exercise (unadjusted and

adjusted for insulin). UcOC level post-exercise was also associated with insulin sensitivity post-exercise although the change in ucOC was not associated with the change in insulin sensitivity post-exercise. The reason for these conflicting results is not clear. It is possible that those with higher baseline ucOC levels have higher insulin sensitivity at rest and post-exercise independent of the relative change in ucOC post exercise. A confirmation for this hypothesis is the moderate, but significant, increase in ucOC post-exercise, compared to a large change in insulin sensitivity. Hence, the results of the current study suggest that individuals with higher basal ucOC are more likely to have higher insulin sensitivity at rest and post-exercise, independent of the change in ucOC post exercise.

Currently, it is unknown whether ucOC has a direct or indirect effect in the insulin sensing effects of exercise. An indirect pathway by which exercise may improve glycaemic control via increases in ucOC is the ucOC effect on islet cells ⁽⁶⁾ and/or increase insulin sensitivity ⁽⁸⁾ that in turn may increase glucose uptake by skeletal muscle. A direct effect of ucOC on skeletal muscle is also plausible although the ucOC receptor in skeletal muscle has yet to be identified. The G protein-coupled receptor 6A (GPCR6A) may be an ucOC receptor in skeletal muscle as it is the ucOC receptor in the Leydig cells of the testes, as well as in pancreatic beta-cells and perhaps in adipocytes and in the liver ⁽²⁶⁻²⁸⁾. The GPCR6A receptor has been identified in skeletal muscle and is implicated as a modulator of glucose metabolism ⁽²⁹⁾. GPCR6A ^{-/-} mice have glucose intolerance ⁽³⁰⁾. Further studies are needed to explore these mechanisms, as well as whether the GPCR6A is in fact the ucOC receptor in skeletal muscle.

The current study report that exercise increases p-AKT and p-AS160. This is important as both proteins are downstream proteins in the PI3-K pathway regulating GLUT-4 translocation that in turn may increase the muscle capacity to take up more glucose ^(14, 15). The current study result indicate that serum ucOC level following exercise was associated with the change in p-AKT following exercise and insulin. It is possible that the pathway by which ucOC increases insulin sensitivity, directly or indirectly, is by activating p-AKT and p-AS160. All together, in humans, similar to mice, ucOC may be partly involved in insulin sensitivity.

A potential limitation of the study is the small sample size. Yet, this sample size was used previously in invasive study that includes several muscle biopsies and two euglycemic-hyperinsulinemic clamps, across two sessions, for each participant ^(31, 32). The study was powered to detect changes in ucOC, as well as in insulin sensitivity. In addition, we did not assess vitamin K intake which may affect the carboxylation of OC.

However, we have no reason to suspect that this may affect the results of the current study as the ucOC levels at baseline between the two sessions were similar.

In conclusion, acute high intensity exercise increases ucOC levels and improves insulin sensitivity post-exercise. UcOC was associated with insulin sensitivity at rest and post-exercise. Insulin sensitivity, at rest and following acute exercise, is associated with circulating levels of ucOC in obese men. Whether ucOC has a direct effect on skeletal muscle insulin sensitivity after exercise is yet to be determined.

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References

1. Holloszy JO. Exercise-induced increase in muscle insulin sensitivity. *J Appl Physiol.* 2005;99(1):338-43.
2. Funai K, Schweitzer GG, Sharma N, Kanzaki M, Cartee GD. Increased AS160 phosphorylation, but not TBC1D1 phosphorylation, with increased postexercise insulin sensitivity in rat skeletal muscle. *Am J Physiol Endocrinol Metab.* 2009;297(1):E242-51.
3. Oguri M, Adachi H, Ohno T, Oshima S, Kurabayashi M. Effect of a single bout of moderate exercise on glucose uptake in type 2 diabetes mellitus. *J Cardiol.* 2009;53(1):8-14.
4. Wojtaszewski JF, Richter EA. Effects of acute exercise and training on insulin action and sensitivity: focus on molecular mechanisms in muscle. *Essays in biochemistry.* 2006;42:31-46.
5. Confavreux CB, Levine RL, Karsenty G. A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. *Mol Cell Endocrinol.* 2009;310(1-2):21-9.
6. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. *Cell.* 2007;130(3):456-69.
7. Booth SL, Centi A, Smith SR, Gundberg C. The role of osteocalcin in human glucose metabolism: marker or mediator? *Nature reviews Endocrinology.* 2013;9(1):43-55.
8. Fernandez-Real JM, Izquierdo M, Ortega F, et al. The relationship of serum osteocalcin concentration to insulin secretion, sensitivity, and disposal with hypocaloric diet and resistance training. *J Clin Endocrinol Metab.* 2009;94(1):237-45.
9. Kanazawa I, Yamaguchi T, Yamamoto M, et al. Serum osteocalcin level is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus. *J Clin Endocrinol Metab.* 2009;94(1):45-9.
10. 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.

11. Levinger I, Scott D, Nicholson GC, et al. Undercarboxylated osteocalcin, muscle strength and indices of bone health in older women. *Bone*. 2014.
12. Hwang YC, Jeong IK, Ahn KJ, Chung HY. The uncarboxylated form of osteocalcin is associated with improved glucose tolerance and enhanced beta-cell function in middle-aged male subjects. *Diabetes Metab Res Rev*. 2009;25(8):768-72.
13. 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.
14. Cartee GD, Wojtaszewski JF. Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Applied Physiology, Nutrition, and Metabolism* 2007;32(3):557-66.
15. Krook A, Wallberg-Henriksson H, Zierath JR. Sending the signal: molecular mechanisms regulating glucose uptake. *Medicine and Science in Sports and Exercise*. 2004;36(7):1212-7.
16. Levinger I, Goodman C, Hare DL, Jerums G, Selig S. The effect of resistance training on functional capacity and quality of life in individuals with high and low numbers of metabolic risk factors. *Diabetes Care*. 2007;30(9):2205-10.
17. Hutchison SK, Stepto NK, Harrison CL, Moran LJ, Strauss BJ, Teede HJ. Effects of exercise on insulin resistance and body composition in overweight and obese women with and without polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2011;96(1):E48-56.
18. Stepto NK, Cassar S, Joham AE, et al. Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulinaemic clamp. *Human reproduction*. 2013;28(3):777-84.
19. 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.
20. Evans WJ, Phinney SK, Young VR. Suction applied to a muscle biopsy maximizes sample size. *Medicine and Science in Sports and Exercise*. 1982;14:101-2.

21. 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.
22. Rong H, Berg U, Topping O, Sundberg CJ, Granberg B, Bucht E. Effect of acute endurance and strength exercise on circulating calcium-regulating hormones and bone markers in young healthy males. *Scand J Med Sci Sports.* 1997;7(3):152-9.
23. Grimston SK, Tanguay KE, Gundberg CM, Hanley DA. The calciotropic hormone response to changes in serum calcium during exercise in female long distance runners. *J Clin Endocrinol Metab.* 1993;76(4):867-72.
24. Welsh L, Rutherford OM, James I, Crowley C, Comer M, Wolman R. The acute effects of exercise on bone turnover. *International journal of sports medicine.* 1997;18(4):247-51.
25. Tosun A, Bolukbasi N, Cingi E, Beyazova M, Unlu M. Acute effects of a single session of aerobic exercise with or without weight-lifting on bone turnover in healthy young women. *Mod Rheumatol.* 2006;16(5):300-4.
26. Oury F, Ferron M, Huizhen W, et al. Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis. *J Clin Invest.* 2013;123(6):2421-33.
27. Pi M, Quarles LD. Multiligand specificity and wide tissue expression of GPRC6A reveals new endocrine networks. *Endocrinology.* 2012;153(5):2062-9.
28. Wei J, Hanna T, Suda N, Karsenty G, Ducy P. Osteocalcin Promotes beta-Cell Proliferation During Development and Adulthood Through Gprc6a. *Diabetes.* 2014;63(3):1021-31.
29. Jean-Baptiste G, Yang Z, Khoury C, Gaudio S, Greenwood MT. Peptide and non-peptide G-protein coupled receptors (GPCRs) in skeletal muscle. *Peptides.* 2005;26(8):1528-36.
30. Pi M, Chen L, Huang MZ, et al. GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. *PloS one.* 2008;3(12):e3858.

31. Stepto NK, Benzi B, Wadley GD, et al. Short-term intensified cycle training alters acute and chronic responses of PGC1alpha and Cytochrome C oxidase IV to exercise in human skeletal muscle. PloS one. 2012;7(12):e53080.
32. Howlett K, Sakamoto K, Garnham A, Cameron-Smith D, Hargreaves M. Resistance exercise and insulin regulate AS160 and interaction with 14-3-3 in human skeletal muscle. Diabetes. 2007;56:1608-14.

Figures Legends

Figure 1. Experimental design. Two muscle biopsies were obtained during the Rest-control session (pre and post insulin clamp) and three muscle biopsies were obtained during the Exercise session (at baseline and pre and post insulin clamp).

Figure 2. Acute high intensity aerobic exercise increases ucOC (undercarboxylated OC) and ucOC/OC ratio, but not tOC (total osteocalcin).

Figure 3. Acute aerobic exercise reduces blood glucose during the hour post-exercise and increases insulin sensitivity 3h post-exercise. GIR (glucose infusion rate, ml/kg/min), M-value (GIR per unit of serum insulin).

Figure 4. Acute high intensity aerobic exercise and insulin infusion have no significant effect on total AKT (panel A) total AS160 (Panel C), but it increases phosphorylated AKT (Panel B) and phosphorylated AS160 (panel D).

Table 1. characteristics of participants (Mean±SEM)

	Mean±SEM
Age (yrs)	58.1±2.2
Height (cm)	176±1.7
Mass (kg)	102.5±3.9
BMI (kg·m ⁻²)	33.1±1.4
Fasting Glucose (mmol·L ⁻¹)	5.3±0.2
HbA1c (%)	5.6±0.1
Trig (mmol·L ⁻¹)	1.9±0.5
HDL (mmol·L ⁻¹)	1.3±0.1
SBP (mmHg)	133.4±3.6
DBP (mmHg)	84.5±1.8
25OHD (nmol/L)	72.9±7.6
VO _{2peak} (ml·kg ⁻¹ ·min ⁻¹)	23.0±1.3
VO _{2peak} (ml·min ⁻¹)	2342±160.8

BMI (body mass index), HbA1c (glycosylated haemoglobin), Trig (triglyceride), HDL (high density lipoprotein), SBP (systolic blood pressure), DBP (diastolic blood pressure).

Table 2. Baseline comparisons between the Rest-control session and the Exercise session

	Rest-control	Exercise
Glucose (mmol/L)	5.2±0.2	5.3± 0.3
Insulin (μU/mL)	13.1±2.4	12.4±2.4
tOC(ng/mL)	17.5±1.4	18.2±1.4
ucOC (ng/mL)	10.8±0.8	10.6±0.8
UcOC/OC ratio	60.8±1.7	58.9±2.0

tOC (total osteocalcin), UCoc (undercarboxylated osteocalcin). Data presented as mean±SEM. All comparisons p>0.05.

Figure 1

Figure 2

Figure 3

Figure 4