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Hydroxytyrosol modifies skeletal muscle GLUT4/AKT/Rac1 axis in trained rats

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Abstract

Training induces a number of healthy effects including a rise in skeletal muscle (SKM) glucose uptake. These adaptations are at least in part due to the reactive oxygen species produced within SKM, which is in agreement with the notion that antioxidant supplementation blunts some training-induced adaptations. Here, we tested whether hydroxytyrosol (HT), the main polyphenol of olive oil, would modify the molecular regulators of glucose uptake when HT is supplemented during exercise. Rats were included into sedentary and exercised (EXE) groups. EXE group was further divided into a group consuming a low HT dose (0.31 mg·kg·d; EXElow), a moderate HT dose (4.61 mg·kg·d; EXEmid), and a control group (EXE). EXE raised glucose transporter type 4 (GLUT4) protein content, Ras-related C3 botulinum toxin substrate 1 (Rac1) activity, and protein kinase b (AKT) phosphorylation in SKM. Furthermore, EXElow blunted GLUT4 protein content and AKT phosphorylation while EXEmid showed a downregulation of the GLUT4/AKT/Rac1 axis. Hence, a low-to-moderate dose of HT, when it is supplemented as an isolated compound, might alter the beneficial effect of training on basal AKT phosphorylation and Rac1 activity in rats.

KEYWORDS
antioxidants, exercise, glucose, polyphenols, skeletal muscle

1 | INTRODUCTION

Glucose uptake during skeletal muscle (SKM) contraction is mediated by GLUT4 translocation to the cellular membrane (Kristiansen, Hargreaves, & Richter, 1997). Contraction-induced GLUT4 translocation can be produced via protein kinase b (AKT) and/or Rac1 (Richter & Hargreaves, 2013; Sano et al., 2003; Sylow et al., 2016). Moreover, chronic exercise (i.e., training) increases GLUT4 protein content, thereby increasing glucose uptake by SKM (Ritcher, 2013); but in spite of that, AKT phosphorylation and Rac1 activity has been mainly studied in response to acute exercise. While 4 weeks of endurance running increases AKT phosphorylation in rat's heart (Jia, Hou, Lv, Xi, & Tian, 2019), 7 days of low-frequency electrical stimulation raises AKT content but not AKT phosphorylation in SKM (Ljubicic & Hood, 2009). Therefore, it is currently unclear whether training increases basal Rac1 activity and AKT phosphorylation within SKM.

Olive oil intake improves glucose uptake in subjects with type 2 diabetes (Schwingshackl et al., 2017). The main polyphenol found in olive oil is hydroxytyrosol (HT) which at doses of ~20 mg·kg·d is highly antioxidant (Cao et al., 2014) and blunts endurance capacity in trained rats (Al Fazazi, Casuso, Aragón-Vela, Casals, & Huertas, 2018). In contrast, high-dose olive oil intake improves exercise performance (Esquius, Garcia-Retortillo, Balagué, Hristovski, & Javierre, 2019) and enhances cellular membrane integrity during training (Quiles et al., 1999), which lead us to hypothesize that a physiological dose of HT intake may preserve SKM adaptations to training.

In this context, training-mediated glucose uptake enhancement seems to be dependent upon reactive oxygen species (ROS) production as antioxidant intake prevents such a training effect (Ristow

et al., 2009). Furthermore, acute exercise-induced AKT phosphorylation and Rac1 activity is dependent upon ROS produced within contracting muscles (Henríquez-Olguin et al., 2019; Parker, Shaw, Stepto, & Levinger, 2017). Therefore, the present study aims to study (a) the effects of training on basal AKT phosphorylation and Rac1 activity and (b) whether a low-to-moderate HT intake alters the adaptation on the GLUT4/AKT/Rac1 axis in SKM of trained rats.

2 | METHODS

2.1 | Animals

Male Wistar rats were purchased from Charles River (Wilmington, MA) at 6 weeks old. The rats initially weighed 200 ± 15.8 g and were maintained in a well-ventilated room under standard conditions. All interventions lasted for 10 weeks. Rats were randomly allocated into a sedentary ($n = 6$) or exercised (EXE) groups ($n = 18$) for 10 weeks. Exercised groups were divided into three groups (EXE, $n = 6$; low HT dose [EXElow], $n = 6$; moderate HT dose [EXEmid], $n = 6$). Exercise training and testing has been previously described (Al Fazazi et al., 2018). Briefly, rats performed a 10-week running training period where they run 5 days/week at 70% of their maximal velocity, which was adjusted after 5 weeks of training. Maximal velocity was assessed using an incremental intensity running protocol where the rats started running at a velocity of 22 cm/s and which was increased by 5 cm/s every minute. Fatigue was considered as the inability of the rat to maintain the pace. Seventy-two hours after the last exercise was performed, rats were fasted overnight, anesthetized with pentobarbital, and killed by bleeding. The soleus muscle was harvested for analysis. The experiments were approved by the ethics committee of the University of Granada (Granada, Spain; no: 28/06/2016/116).

2.2 | HT treatment

EXElow animals were supplemented with 0.31 mg·kg·d of HT while EXEmid group received a dose of HT (4.61 mg·kg·d). This is the minimum dose range able to rise total plasma antioxidant capacity (Cicerale, Lucas, & Keast, 2010). The low HT dose is similar to the daily HT intake from extra virgin olive oil in the Spanish population (Martínez, Ros, & Nieto, 2018), which it is known to consume 30–50 g of extra virgin olive oil daily (Tresserra-Rimbau et al., 2013). However, the moderate HT dose could only be achieved by nutraceutical enrichment, as it would require ~0.6 L of extra virgin olive oil daily consumption.

An aqueous virgin olive extract rich in HT (15%) was diluted in water in an opaque drinking bottle to prevent oxidation. The dilution was adjusted weekly according to the weight of each rat and its average water intake. Further details on the supplementation and extraction protocol can be found elsewhere (Rodríguez-Lara et al., 2019). Supplementation stopped 12 hr before rats were euthanized.

2.3 | Quantitative real-time polymerase chain reaction

We used the Real-Time Ready Custom Panel 96 (Roche, Barcelona, Spain), which is a two-step quantitative real-time polymerase chain reaction (qRT-PCR) platform, which has been previously described in detail (Huertas et al., 2019). The Real-Time Ready Custom Panel 96 (Roche) included the following specific primer pairs: *Slc2a4* (Assay ID 500810; Roche, Barcelona, Spain). The expression level of each gene was analyzed with RT^2 Profiler PCR Array Data Analysis software (version 3.4, SABiosciences). Changes in gene expression were expressed as fold changes (Fc) using the sedentary animals as a control.

2.4 | Western blot

Samples were processed as we previously described (Huertas et al., 2019) and the membranes were probed with the following antibodies: anti-GLUT4 (sc-53566; 1:100 in 5% nonfat milk), anti-AKT (C67E7), and anti-phospho-AKT (Ser473, D9E) both 1:1,000 in 5% bovine serum albumin were acquired from Cell Signalling Technologies (Beverly, MA), anti-Hsp-70 (sc-7298; 1:500 in 5% nonfat milk).

2.5 | Rac1 activation assay

Rac1 activity was measured in the supernatant of muscle lysates using a commercially available Rac1 Activation Assay Kit (ab211161). In short, lysates were incubated with agarose beads to selectively isolate and pull-down the active form of Rac (GTP-Rac) from samples. GTP-Rac was detected by western blot analysis using an anti-Rac1 specific monoclonal antibody, which is provided by the kit.

2.6 | Lipid peroxidation and protein carbonyls in the plasma

Blood was collected and centrifuged for 10 min at 3,000 rpm to isolate the plasma. Then, 40 μl of plasma was used to quantify hydroperoxides concentration and protein carbonyls adducts. The concentration of hydroperoxides was determined using a Sigma PD1 Kit (St Louis, MO). Absorbance changes at 560 nm were monitored by spectrophotometry. Protein carbonyls were measured using an enzyme-linked immunosorbent assay-based assay according to manufacturer's instructions (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs, Inc., San Diego, CA).

2.7 | Statistical analysis

Results are shown as the mean ± standard deviation. Homoscedasticity and normality were tested by Levene's test and the Kolmogorov–Smirnov test, respectively. One-way analysis of

variance was used to analyze the data. A post hoc analysis was performed, confidence intervals were adjusted using Bonferroni correction when the effect was statistically significant. The level of significance was set at $p < .05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 22 for Windows; IBM Corp., Armonk, NY).

3 | RESULTS

We observed that trained animals showed higher maximal running velocity and lower weight gain than sedentary rats. However, HT did not alter these parameters when supplemented during exercise (Table 1). Moreover, plasma protein carbonyls and hydroperoxides levels were similar between experimental groups (Table 2).

GLUT4 messenger RNA (mRNA) levels tended to raise in the soleus of EXE animals but this effect was significant in EXEmid, while no effect was detected in EXElow rats (Figure 1a). However, at the protein level, GLUT4 raised only in EXE animals (Figure 1b). Similarly, we found that both the low and the moderate HT doses altered AKT phosphorylation as denoted by the phospho-AKT (Ser473)/total AKT ratio (Figure 1c). We next assessed the Rac1 activity as a possible compensatory mechanism for GLUT4 translocation to the plasma membrane independently from AKT (Raun et al., 2018). Notably, we found that exercise increased Rac1 activity in SKM and that this effect was maintained in EXElow but hampered in EXEmid (Figure 1d).

4 | DISCUSSION

SKM adaptations to training are highly dependent upon ROS production within contracting muscles. Furthermore, olive oil consumption improves glucose uptake in type 2 diabetes subjects (Schwingshackl et al., 2017) and it improves the acute response to exercise in rats and humans (Esquius et al., 2019; Quiles et al., 1999). Then, in the present study, we tested whether low-to-moderate dose of HT, when it is supplemented during training, alters the molecular regulators of glucose uptake. Our results show that 10 weeks of running raise basal AKT phosphorylation at Ser473 and Rac1 activity as well as GLUT4 protein content, whereas HT supplementation blunts those adaptations in a dose-dependent manner.

Deleterious effects on exercise performance have been reported in rodents consuming 20 mg·kg·d (Al Fazazi et al., 2018), a dose with high antioxidant potential within SKM (Cao et al., 2014). Notably, here we used significant lower HT doses that are known to be able to rise plasma antioxidant capacity. However, we have been unable to detect differences in plasma oxidative stress markers, suggesting that the methods used may not be sensitive enough to detect changes in plasma oxidative status in healthy trained rats, and may not reflect SKM oxidative stress. Nevertheless, our data are in line with previous findings where training proceeded as an antioxidant, thus antioxidant supplementation hampers some of its adaptations (Gomez-Cabrera et al., 2009; Gomez-Cabrera, Domenech, & Viña, 2008). In fact, our results are in line with the notion that antioxidant supplementation can blunt the effects of training on the molecular regulators of glucose uptake (Ristow et al., 2009) and may prevent AKT activation (Parker et al., 2017). In addition, it is known that Rac1 controls glucose uptake during exercise in an ROS-dependent manner (Henríquez-Olguin et al., 2019). Here, we report for the first time that antioxidant supplementation prevents the training-induced adaptation on AKT phosphorylation at the Ser473 site and Rac1 activity. It should be noted that Rac1 controls SKM glucose uptake within exercised muscle by promoting GLUT4 translocation to the plasma membrane (Sylow et al., 2016). In fact, glucose uptake via Rac1 occurs independently from AKT activity (Raun et al., 2018). Importantly, we have not detected changes in maximal exercise capacity, which may indicate that (a) low-to-moderate HT doses hampers exercise-induced basal glucose metabolism but not glucose uptake during exercise, and/or that (b) the blunted molecular signaling regulating glucose uptake precedes the loss of exercise capacity. Moreover, we observed that the moderate HT dose blunts the entire AKT/Rac1 axis, whereas the low dose only hampers AKT phosphorylation. Thus, suggesting that Rac1 activity may be essential for basal glucose uptake as it appears to be less sensitive to mild SKM homeostatic changes.

An alternative explanation for the similar exercise performance between experimental groups can be provided by the exercise protocol. We applied a protocol where running velocity was increased every minute until exhaustion (~12–15 min). This kind of exercise may compromise both glycolytic and oxidative metabolic pathways (Huertas, Casuso, Agustin, & Cogliati, 2019). Therefore, energy supply from fatty acids oxidation may compensate for the alterations (if any) in glucose oxidation.

TABLE 1 Physical performance and weight gain

	Control	EXE	HTlow	HTmid
Preliminary maximal velocity (cm/s)	76.4 ± 5.9	77.8 ± 5.8	76.2 ± 8.0	75.2 ± 5.1
Final maximal velocity (cm/s)	65.3 ± 7.2*	92.3 ± 7.6*,**	86.6 ± 13.8*,**	93.8 ± 7.9*,**
Weight gain (g)	216 ± 42.3	126 ± 26.5**	118 ± 19.5**	134 ± 23.7**

Note: Data are shown as mean ± *SD*; $n = 6$.
Abbreviations: EXE, exercised; HTlow, exercised and supplemented with 0.31 mg·kg·d of hydroxytyrosol; HTmid, exercised and supplemented with 4.61 mg·kg·d of hydroxytyrosol; *SD*, standard deviation.
*Statistically different versus preliminary measurement.
**Statistically different versus controls.

TABLE 2 Plasma oxidative stress markers

	Control	EXE	HTlow	HTmid
Hydroperoxides (nmol/ml)	3.80 ± 0.82	3.2 ± 0.51	3.1 ± 0.62	3.9 ± 1.17
Protein carbonyls (nmol/mg)	0.12 ± 0.023	0.15 ± 0.073	0.12 ± 0.025	0.11 ± 0.008

Note: Data are shown as mean ± *SD*; *n* = 6.
Abbreviations: EXE, exercised; HTlow, exercised and supplemented with 0.31 mg·kg·d of hydroxytyrosol; HTmid, exercised and supplemented with 4.61 mg·kg·d of hydroxytyrosol; *SD*, standard deviation.

It should be noted that we observed that GLUT4 mRNA significantly raised in response to a moderate dose of HT together with a numerical but not significant increase in GLUT4 protein content. This is a conspicuous finding as ROS induces GLUT4 transcription through p38 mitogen-activated protein kinase (Richter & Hargreaves, 2013). In this regard, the main limitation of the present study is that we have not quantified SKM oxidative status. However, the redox reactions produced in living cells following polyphenol

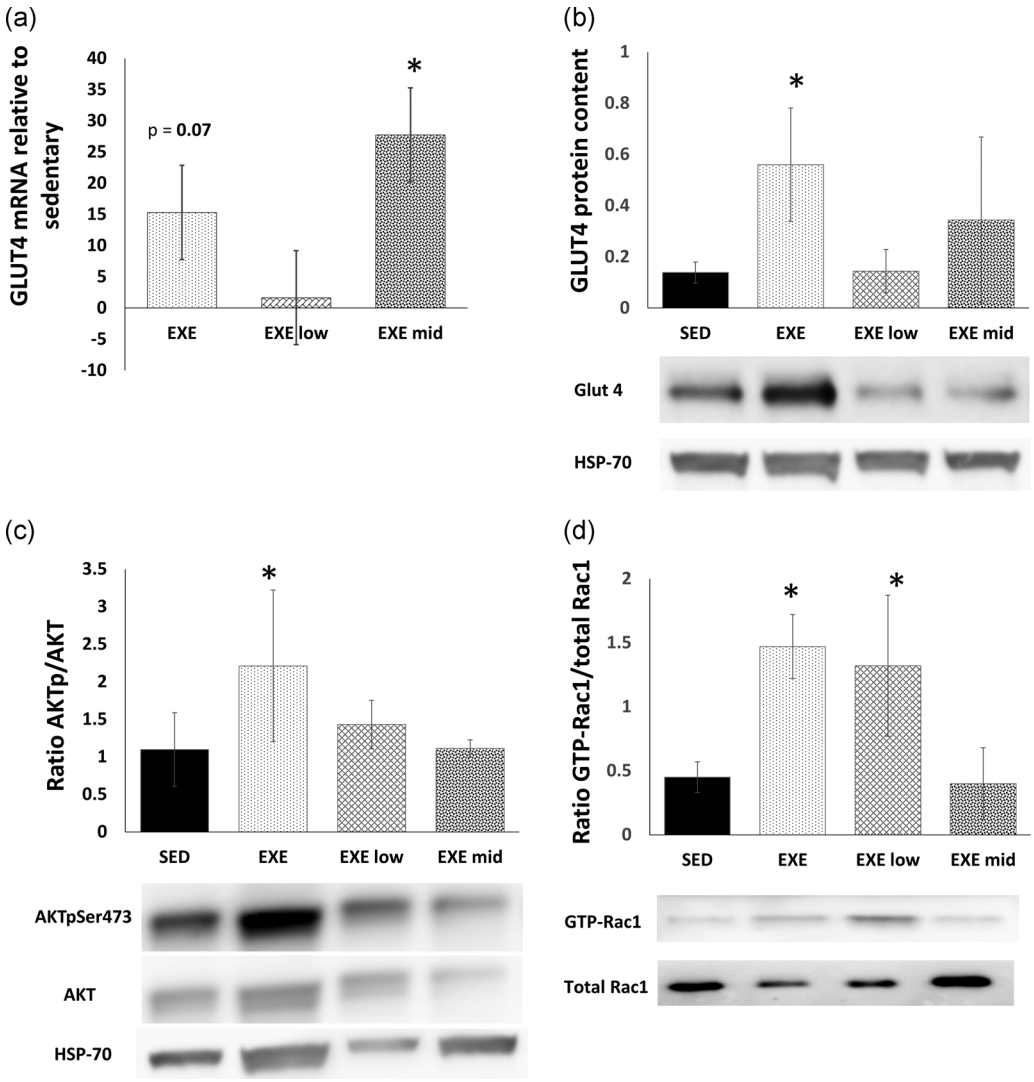

FIGURE 1 Molecular regulators of glucose uptake within soleus muscle. (a) Moderate hydroxytyrosol dose increases GLUT4 mRNA levels in exercised animals. RNA data are shown as fold change relative to the sedentary group. (b) GLUT4 protein content raises following 10 weeks of training. (c) Both a low and a moderate hydroxytyrosol dose blunts the exercise-induced increment of the ratio between AKT phosphorylation at Ser^{473} (AKTp) and total AKT. (d) Exercise increases Rac1 activation. This effect is maintained when a low HT intake is supplemented but hampered when a moderate HT dose is consumed. All the analyses were conducted in six animals per group. *$p < .05$ if compared with SED. AKT, protein kinase b; EXE, exercised animals; EXElow, exercised and supplemented with a low hydroxytyrosol dose (i.e., 0.31 mg·kg·d); EXEmid, exercised and supplemented with a low hydroxytyrosol dose (i.e., 4.61 mg·kg·d); HT, hydroxytyrosol; mRNA, messenger RNA; SED, sedentary animals

supplementation are poorly understood. For instance, following its antioxidant activity, some of the metabolites produced became pro-oxidant (Boots et al., 2007). This pro-oxidant effect has been reported in exercised rats supplemented with 12 mg·kg·d of quercetin (Casuso et al., 2015). On the other hand, in vitro experiments show that low HT doses ranging from 0.1 to 10 μM can modulate mitochondrial metabolism by activating PPARG coactivator 1-α promoter activity (Hao et al., 2010). Thus, it is possible that a moderate HT dose might modulate GLUT4 gene expression through epigenetic mechanisms and/or by redox mechanisms.

The present study has several limitations that should be addressed in future studies. For instance, exercise training itself acts as an antioxidant that may mask any antioxidant effect induced by the HT doses applied. Therefore, the antioxidant potential of low-to-moderate HT doses should be tested in sedentary and/or insulin-resistant rodents. This may help to mechanistically link the blunted AKT and Rac1 response to training with an antioxidant effect of HT. Moreover, given the rising importance of Rac1 in glucose uptake during exercise, future studies should test some downstream markers of Rac1 activity both at rest and during exercise following antioxidant supplementation. Finally, it is important to highlight that HT can be differently metabolized between rats and humans. Therefore, our results should be viewed with caution and future studies must confirm our observations in human SKM.

In conclusion, here we describe that exercise increases basal Rac1 activity as well as GLUT4 protein content and AKT phosphorylation at Ser473. Moreover, we found that very low doses of HT blunt the AKT and Rac1 axis. This effect seems to be dose-dependent as 0.31 mg·kg·d prevents AKT phosphorylation while 4.31 mg·kg·d prevents both AKT phosphorylation and Rac1 activation. Therefore, exercising subjects need to consider that intake of isolated antioxidant compounds, even at very low doses, might alter some of the beneficial effects of exercise training.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

R. A. C. and J. R. H. conceptualized and designed the study. S. A. F., J. A.-V., F. J. R.-O., J. P.-D., A. R.-R. contributed to the acquisition of data. R. A. C., J. R. H., J. A.-V., F. J. R.-O. contributed to the analysis and interpretation. R. A. C. and S. A. F. drafted the article. All the authors approved the final version.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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