Published in final edited form as:

Clin Sci (Lond). 2013 June ; 124(11): 663–674. doi:10.1042/CS20120469.

(–)-EPICATECHIN IS ASSOCIATED WITH INCREASED ANGIOGENIC AND MITOCHONDRIAL SIGNALING IN THE HINDLIMB OF RATS SELECTIVELY BRED FOR INNATE LOW RUNNING CAPACITY

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Abstract

Alternative approaches to reduce congenital muscle dysfunction are needed in cases where the ability to exercise is limited. (–)-Epicatechin is found in cacao and may stimulate capillarity and mitochondrial proliferation in skeletal muscle. Twenty-one male rats bred for low running capacity (LCR) from generation 28 were randomized into three groups: vehicle for 30 days (control); (–)-epicatechin for 30 days; and (–)-epicatechin for 30 days followed by 15 days without (–)-epicatechin. Groups 2 and 3 received 1.0 mg/kg of body weight of (–)-epicatechin twice daily, whereas water was given to the control group. The plantaris muscle was harvested for protein and morphometric analyses. In addition, *in vitro* experiments were conducted to examine the role of (–)-epicatechin on mitochondrial respiratory kinetics at different incubation periods. Thirty days of (–)-epicatechin treatment increased capillarity ($p < 0.001$) and was associated with increases in protein expression of VEGF-A with a concomitant decrease in TSP-1 and its receptor, which remained after 15 days of (–)-epicatechin cessation. Analyses of p38 MAPK signaling pathway indicated an associated increase in phosphorylation of MKK3/6 and p38 and increased protein expression of MEF2A. In addition, we observed significant increases in protein expression of PGC-1 α , PGC-1 β , and Tfam, and cristae abundance. Interestingly, these increases associated with (–)-epicatechin treatment remained after 15 days of cessation. Lastly, *in vitro* experiments indicated that acute expose of LCR muscle to (–)-epicatechin incubation was not sufficient to increase mitochondrial respiration. The results suggest increases in skeletal muscle capillarity and mitochondrial biogenesis are associated with 30 days of (–)-epicatechin treatment and sustained 15 days following cessation of treatment. Clinically, the use of this natural compound may have

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potential application in populations that experience muscle fatigue and are unable to perform endurance exercise.

Keywords

Dark chocolate; Natural compounds; Nutrition; Supplementation

INTRODUCTION

Although exercise results in many health benefits there are numerous circumstances in which the ability to exercise is limited. These situations may originate from genetic malfunction such as mitochondrial myopathy or prolonged bed rest, for example after physical trauma or during chemotherapy. The physiological adaptations to endurance training results in multi-level changes to the working muscle [1]. For example, at the molecular level activation of the p38 mitogen-activated protein kinase (MAPK) pathway leads to mitochondrial biogenesis via increases in peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) proteins [2, 3]. At the skeletal muscle level, studies have shown that capillary proliferation is due to increases in vascular endothelial growth factor (VEGF-A) with a concomitant decrease in thrombospondin-1 (TSP-1) [4–6]. The inability to exercise the muscle, therefore, results in deconditioning which leads to muscle dysfunction and thus fatigue [4].

Investigators have examined the exercise-mimetic effects of numerous compounds [7, 8]. Narkar and colleagues [7] reported that sedentary mice given GW501516, which increases the uptake of glucose, did not result in exercise-mimetic effects in the hindlimb muscles, whereas Kleiner and colleagues [9] concluded that this compound did not stimulate mitochondrial biogenesis. Jackson et al. [8] reported that resveratrol did not attenuate sarcopenia in middle-aged mice. Our laboratory, recently, has focused on a flavanol compound derived from the cocoa seed called (–)-epicatechin to improve skeletal muscle angiogenesis and mitochondrial biogenesis [4, 10]. In addition, we found that in healthy middle-aged mice (–)-epicatechin results in increased oxidative capacity independent of endurance exercise [10]. Furthermore, Hüttemann et al. [4] reported that mice administered (–)-epicatechin maintained the 8-week endurance training-induced capillarity and oxidative phosphorylation following 14 days of detraining compared to mice receiving the placebo. These findings [4, 10] suggest that (–)-epicatechin may have exercise-mimetic properties, however, the underlying mechanism for these improvement in skeletal muscle is unclear. Furthermore, the continued effects of (–)-epicatechin on skeletal muscle are not known after cessation of the compound, because the bioavailability of (–)-epicatechin 24 hours following administration has been shown to be low in rodent models [11, 12].

To examine the genomic factors mechanistically responsible for decreasing fatigue and increasing oxidative capacity, Koch and Britton [13] developed a contrasting animal model system by two-way artificial selection for innate treadmill running ability using a heterogeneous rat stock (N:NIH) as the founding population. After 6 generations of selection, two distinct lines of low and high capacity runners (called LCR and HCR, respectively) with a 171% difference in endurance capacity emerged [13]. With strong selection, the difference in endurance capacity increased to 347% by generation 11 [14] and 414% by generation 20 [15]. Studies examining oxygen transport and utilization components of the hindlimb muscles in LCR and HCR rats have shown a significant divergence in oxygen extraction and utilization which are related to factors such as capillarity and oxidative phosphorylation impairment [16–18]. Kivelä et al. [19] used microarray analysis and reported that the hindlimb muscles of LCR rats had reduced

expression of genes related to impaired oxidative metabolism that highly correlated with disease risk factors compared to HCR rats. Furthermore, in healthy rats, (–)-epicatechin has been shown to have cardioprotective properties during ischemic conditions [20, 21]. Therefore, the LCR rats present a unique opportunity to examine innate skeletal muscle dysfunction which may be indicative of congenital conditions such as mitochondrial myopathy.

Since outbred selected lines maintain genetic complexity, the LCR line of rats present a model to examine the effects of (–)-epicatechin better suited for human translation. The purposes of this study were to conduct a 30 day treatment of LCR rats with (–)-epicatechin and determine whether (–)-epicatechin can 1) increase skeletal muscle capillarity by regulating protein expression of VEGF-A and TSP-1; 2) increase mitochondrial biogenesis by regulating key promoters such as PGC-1 α , PGC-1 β , and Tfam; 3) increase mitochondrial respiratory kinetics using an *in vitro* approach; and 4) maintain these improvements after 15 days of (–)-epicatechin cessation. We hypothesized that (–)-epicatechin would increase skeletal muscle capillarity when compared to vehicle control. In addition, we hypothesized that the (–)-epicatechin-treated LCR rats would have increased oxidative capacity by increasing mitochondrial biogenesis and cytochrome *c* oxidase (CcO) protein expression. Furthermore, we hypothesized that these improvements in skeletal muscle capillarity and oxidative capacity would return to control levels after cessation of (–)-epicatechin for 15 days.

METHODS

Animals and housing

We studied 5-month old, male LCR rats (N = 21) from generation 28. Animals were placed two per cage and fed a standard rodent chow diet without limitations. Standard animal housing was used (21 °C with 12-hour light:dark cycles). All animal care and experimental procedures were approved by Wayne State University Animal Care and Use Committee.

Experimental Design

A between-subjects design was used to determine the effects of (–)-epicatechin on the plantaris muscle of LCR rats. Animals were randomized into three groups (n=7): group 1- vehicle for 30 days (control); group 2- (–)-epicatechin for 30 days ((–)-Epi 30d); and group 3- (–)-epicatechin for 30 days followed by 15 days without (–)-epicatechin (Post (–)-Epi 15d). Twenty-four hours after the final dosage, rats in groups 1 and 2 were sacrificed. Rats in group 3 were sacrificed 15 days after the final dosage. This approach was taken in order to determine how long the effects of (–)-epicatechin would remain in the muscle once treatment stopped. For all three groups, the plantaris muscle was analyzed, because we wanted to determine the effects of (–)-epicatechin on a predominantly glycolytic muscle fiber.

(–)-Epicatechin Administration

Consistent with our previous work on (–)-epicatechin (Sigma-Aldrich, St. Louis, MO) in mice [4, 10], rats in the (–)-epicatechin groups (2 and 3) were given a dosage of 1.0 mg kg of body mass^{–1} twice a day (morning and evening) for 30 consecutive days, whereas animals in the control group received the vehicle (water). Delivery of (–)-epicatechin and vehicle was via oral gavage by experienced personnel.

Tissue Preparation

Consistent with standard procedures in our laboratory [4, 5, 10, 22, 23], animals were anesthetized with sodium pentobarbital (60 mg×kg^{–1} *i.p.*) and the left plantaris muscle was

removed. The animals were euthanized via cardiac removal. The muscle was then sectioned and frozen in precooled isopentane (-140°C) and stored at -80°C until further processing. Transverse $10\text{ }\mu\text{m}$ serial sections were cut on a cryotome (Leica CM 1950; Buffalo Grove, IL) at -20°C and mounted on slides for histochemical analysis, whereas the remaining pieces of plantaris muscle were used for biochemical and molecular analyses..

Capillary Staining and Indices

For all three groups, the plantaris muscle was stained according to Rosenblatt [24]. Consistent with our previous studies [4, 5, 10], muscle sections were viewed under a digital microscope ($20\times$ magnification, Leica DMD108, Buffalo Grove, IL). Quantification of capillaries was performed to determine, 1) the number of capillaries around a fiber (N_{CAF}), 2) the capillary-to-fiber ratio on an individual-fiber basis (C/F_i), and 3) capillary density (CD) which was calculated by using the fiber area as the reference space [25]. The capillary-to-fiber perimeter exchange index (CFPE) was estimated using the method of Hepple et al [26]. Capillarity was quantified on random sections of the muscle which did not have freeze fractures [27]. Fiber cross-sectional area (FCSA) and perimeter (FP) was measured with the image analysis system and commercial software (SigmaScan Pro v. 5.0, Systat Software, Inc., Point Richmond, CA). In addition, the formula of Snyder [28] was used to estimate the maximal diffusion distance (R_{95}).

CcO Specific Activity Measurements

To examine the acute effect of (–)-epicatechin on respiration kinetics the frozen plantaris muscle (50 mg) from Sprague-Dawley and LCR rats not receiving (–)-epicatechin (i.e., control group) were cut into smaller pieces with scissors. This approach is standard procedure in our laboratory for the determination of CcO activity [29]. CcO specific activity was analyzed with a micro Clark-type oxygen electrode in a closed chamber (Oxygraph system, Hansatech, Norfolk, England) at 25°C . Oxygen consumption was recorded on a computer and analyzed with the Oxygraph software. CcO specific activity was defined as consumed O_2 (nmol)/min-mg total protein and normalized to the healthy muscle at 0 min incubation with no (–)-epicatechin [29].

Western Blotting

The western blot analysis procedure was consistent with our previous work [4, 10, 23]. Fifty milligrams of the plantaris muscle was homogenized in a glass tissue grinder with RIPA buffer (Sigma-Aldrich) and protease and phosphatase inhibitor cocktails (PhosSTOP Phosphatase and Complete Protease Inhibitor Cocktail, Roche Applied Science, Indianapolis, IN). Total protein was measured by the bicinchoninic acid method (BCA protein assay kit, Bio-Rad).

Protein samples ($40\text{ }\mu\text{g}$) were incubated at 95°C for 5 min and loaded onto 7.5% (for TSP-1, PGC-1 α , and PGC-1 β) or 12% TGX pre-cast gels (Bio-Rad) and ran for 1-hour at 160 V. Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) was used on the membranes after semi-dry blotting (12 V, 50 minutes, Bio-Rad, Hercules, CA) for 1 h at room temperature. Thereafter, the primary antibody was used for overnight incubation at 4°C with gentle shaking. On day two, membranes were washed 4 times every 5 minutes in Tris buffered saline with Tween 20 (TBST) wash solution. Then, membranes were incubated with secondary antibody for 1 h with the same washing procedure. The Odyssey infrared imaging system was used to quantify blots (Li-Cor Biosciences).

The monoclonal primary antibodies used were TSP-1 (1:500; sc-59886), PGC-1 β (1:1,000; sc-373771), CD47 (1:500; 3847–1, Epitomics), and GAPDH (1:2,000, ab9484). The polyclonal primary antibodies used were MEF2A (1:500; 2080–1, Epitomics), p38 MAPK

(1:1,000; 9212S, cell signaling), p-p38 MAPK^{Thr180/Tyr182} (1:1,000; 9211S, cell signaling), MKK3 (1:1,000; 9238, cell signaling), p-MKK3^{Ser189}/MKK6^{Ser207} (9231; 1:1,000, cell signaling), VEGF (1:500, sc-507), PGC-1 α (1:1,000; AB3242), anti-Tfam (1:1,000; SAB1401383, Sigma-Aldrich), CcO subunit II (1:500; sc-23984), and α -tubulin (1:2,000, cell signaling 2144). The secondary antibodies used were goat anti-mouse IRDye (1:30,000), goat anti-rabbit IRDye (1:30,000), and rabbit anti-goat IRDye (1:30,000) purchased from Li-Cor Biosciences.

Qualification of Mitochondria

Pieces of the plantaris muscle were fixed in 2% paraformaldehyde plus 2.5% glutaraldehyde (Ted Pella, Redding, CA) in 0.1 M sodium cacodylate (pH 7.4) on ice for 24 hours [10]. Specific preparation of the tissues for electron microscopy were consistent with our previous study [10].

Statistical Analyses

One-way ANOVAs were used to compare group means for various dependent variables. Data are reported as mean $\pm$ SEM. For significant overall *F*-ratio, Tukey's *HSD* was used for followed analyzes. Statistical significance was considered for values ($p < 0.05$) and analyzed with standard statistical program (v. 19.0, IBM SPSS, Armonk, NY).

RESULTS

Animals

The results in Table 1 indicate no statistical difference ($p > 0.05$) for anthropometric measurements between groups.

Hindlimb Capillarity

The one-way ANOVA for fiber cross-sectional area (FCSA) and perimeter measurements for the plantaris muscle revealed a significant *F*-ratio for perimeter only. To further examine the differences among the group means, pairwise Tukey's *HSD* follow-up tests were conducted. As shown in Table 2, fiber perimeter was significantly different for the (–)-Epi-30d and post (–)-Epi-15d groups compared to control.

The one-way ANOVA for capillary indices (N_{CAF} , C/F_i , CD, and CFPE) also resulted in significant *F*-ratios. The follow-up tests indicated that (–)-Epi-30d had higher capillarity than the controls (Fig. 1). In addition, the post (–)-Epi 15d group had significantly higher capillarity than the control group, but significantly lower than the (–)-Epi-30d group.

VEGF, TSP-1, and CD47 Protein Expression

Thirty days of (–)-epicatechin treatment significantly increased VEGF-A protein expression compared to control, whereas for TSP-1 protein expression there were significant decreases (Figure 2). Furthermore, (–)-epicatechin treatment significantly reduced CD47, TSP-1 receptor, compared to the control group (Fig. 3).

Mitochondrial Biogenesis

The one-way ANOVA revealed a significant *F*-ratio for various indices of the p38 MAPK signaling pathway and mitochondrial biogenesis (PGC-1 α , PGC-1 β , and Tfam) (Figures 3 and 4). The follow-up analyses for each of these indices indicated significant increases with (–)-epicatechin treatment (Fig. 4). In addition, once (–)-epicatechin was discontinued for 15 days, these increases remained significantly higher than controls. Although the increase for

mitochondrial volume density with (–)-epicatechin was not statistically significant (Fig. 5A, $p = 0.056$), there were significant increases in cristae abundance (Fig. 5B).

Cytochrome c Oxidase (CcO) Protein Expression and Acute Exposure to (–)-Epicatechin

CcO expression as determined with an antibody against subunit II was significantly increased for the (–)-Epi 30d group from control (Fig. 6A and 6B). Mitochondrial respiration can be affected by changes in protein levels and/or by alternations in the CcO phosphorylation state. To test for the latter possibility we incubated rat plantaris muscle tissue with (–)-epicatechin. We found that CcO activity at maximal turnover increased 47% and 27% after 25 min of incubation with (–)-epicatechin in healthy plantaris muscle compared to control tissue at 0 min and 25 min without (–)-epicatechin, respectively (Fig. 6C). Interestingly, tissues derived from the plantaris muscle of LCR rats who did not receive 30 days of (–)-epicatechin treatment showed no increase in CcO activity. Our data indicate that (–)-epicatechin exerts a long-term effect on CcO expression in the muscle of LCR rats, but not an early time-dependent activation effect on CcO.

DISCUSSION

The primary and distinctive findings of the present study are that 30 days of (–)-epicatechin treatment are associated with increased capillarity and mitochondrial biogenesis in the plantaris muscle of rats with an intrinsically low aerobic capacity. We found that changes in capillarity were associated with changes in VEGF-A, TSP-1, and CD47 protein expression, whereas increases in mitochondria were associated with p38 MAPK signaling. In addition, we found increased protein expression of PGC-1 α , PGC-1 β , and Tfam with (–)-epicatechin treatment. Furthermore, we observed an increase in cristae abundance. In addition, *in vitro* experiments revealed that LCR rats have reduced mitochondrial respiration, which was not increased by short-term (25 min incubation) exposure to (–)-epicatechin. Furthermore, we observed increased protein expression of CcO with (–)-epicatechin treatment after 30 days. Importantly, we discovered that these improvements in capillarity and oxidative capacity of the plantaris muscle were sustained 15 days after cessation of (–)-epicatechin and statistically greater than controls. These results suggest that (–)-epicatechin is associated with increases in two critical components of the oxygen transport pathway (capillaries and mitochondria) in the plantaris muscle of rats selectively bred for low aerobic capacity.

The focus of the current investigation was to determine if (–)-epicatechin could improve angiogenesis and mitochondrial proliferation in glycolytic muscle of LCR rats and if the response remained after 15 days of (–)-epicatechin cessation compared to LCR rats not treated with (–)-epicatechin. Therefore, we examined only glycolytic muscle (i.e., plantaris) and not oxidative muscle (i.e., soleus) in the current investigation. We selected the plantaris muscle because Rivas et al. [30] reported no difference ($p > 0.05$) in mitochondrial content between the soleus muscles of HCR and LCR rats. Furthermore, Lessard et al. [31] reported no significant changes in β -HAD (Beta(3)-Hydroxyacyl-CoA Dehydrogenase) and citrate synthase activity in the soleus muscle of LCR sedentary versus LCR endurance-trained rats. Therefore, since (–)-epicatechin has been shown to increase [10] or maintain [4] components of the mitochondria in glycolytic muscles we elected to focus on the same plantaris muscle.

Glycolytic muscle fibers are characterized as having lower capillarity and mitochondrial density than oxidative muscles [32]. Skeletal muscle, however, is adaptable to various interventions such as endurance exercise [4, 10, 33] or genetic manipulation [5, 34, 35] which can stimulate or inhibit capillary proliferation. A number of potential mechanisms may interplay to stimulate angiogenesis in skeletal muscle [4, 5, 34]. Olfert and Birot [36] have suggested that capillary proliferation may be the result of a balance between VEGF-A

and TSP-1 protein expression. Indeed, results of studies examining glycolytic muscles (i.e., plantaris or quadriceps femoris) using different paradigms such as endurance training [4], hindlimb suspension [37], or diabetes [38] indicate a relationship between increased capillarity in the muscle with a concomitant reduction in TSP-1 protein expression. Studies suggest that the inhibition of VEGF receptor-2 (VEGFR2) by TSP-1 may be via activation of the TSP-1 receptor CD47 [39]. Furthermore, the essential role of CD47 has been shown in tissue ischemia by inhibiting the nitric oxide signaling pathway [40, 41]. Specifically, the binding of TSP-1 to CD47 results in downstream inhibition of nitric oxide stimulated sGC (soluble guanylyl cyclase) thus limiting vascular remodeling [41]. Using a skin graft model, Isenberg and colleagues [42] reported that grafts did not survive when transplanted from one wild-type mouse to another wild-type mouse, whereas wild-type mice receiving grafts from TSP-1 null mice resulted in tissue survival. The investigators hypothesized that the lack of suppression by the TSP-1-CD47 interaction on nitric oxide signaling may have contributed to the survivability of the grafts [42]. The activation of CD47 inhibiting downstream signaling of nitric oxide via VEGFR2 has been reported [43, 44], and Kaur et al. [39] found that CD47 prevents autophosphorylation of VEGFR2 at Tyr¹¹⁷⁵ which inhibits the VEGF pathway. It should be noted, however, that suppression of CD47 restored the VEGFR2 phosphorylation [39]. Frazier et al. [45] examined skeletal muscle characteristics of CD47 null mice. The investigators reported that similar to TSP-1 null mice, CD47 null mice had significantly higher capillary density than littermate controls which functionally translated to increased exercise capacity [45]. The results of the above studies indicate an interaction between TSP-1-CD47-VEGF-A and may potentially explain capillary maintenance and/or regression observed in skeletal muscle under various perturbations.

In the present investigation, we examined protein expression of VEGF-A, TSP-1, and CD47 in the plantaris muscle. Thirty days of (–)-epicatechin treatment increased VEGF-A protein expression by ≈40%, whereas TSP-1 was reduced, on average, by ≈35%. Following 15 days of (–)-epicatechin cessation, however, VEGF-A protein expression returned to control levels, whereas TSP-1 levels remained unchanged from the 30 day treatment group. In addition, we examined CD47 to determine if (–)-epicatechin treatment alters protein expression of the TSP-1 receptor. Thirty days of (–)-epicatechin treatment reduced CD47 protein expression by ≈70% compared to controls and was maintained after cessation of (–)-epicatechin. Associated with these changes, capillary indices were significantly reduced in the post (–)-Epi 15d group compared to the treatment group, but significantly higher than the control group. A potential explanation may be that (–)-epicatechin increases protein expression of VEGF-A independent of reducing TSP-1 and CD47 thus amplifying downstream VEGF signaling which results in the initial proliferation of capillaries. In addition, the return of VEGF-A protein expression to control levels after (–)-epicatechin cessation suggests that VEGF-A, TSP-1, and CD47 have different time courses in which they return to basal levels. Additional studies are required to investigate the signaling mechanism of (–)-epicatechin on the VEGF-A-TSP-1-CD47 interaction as well as determine the prolonged effects upon cessation.

Since (–)-epicatechin may be a potential exercise-mimetic compound as reported previously [4, 10], we therefore examined components of the p38 MAPK pathway to better understand the mechanism of (–)-epicatechin-induced mitochondrial biogenesis. We found no change in MKK3 protein expression with (–)-epicatechin treatment, but an increase in p-MKK3/6. Similarly, p38 MAPK protein expression was unchanged, but p-p38 MAPK increased by 234% following 30 days of (–)-epicatechin treatment compared to controls. This increase, however, returned to control levels after 15 days of (–)-epicatechin cessation. We also examined downstream targets of p38 MAPK and found that MEF2A protein expression increased (56%) with (–)-epicatechin treatment.

Studies using transgenic mouse models of PGC-1 α [2] and PGC-1 β [46, 47] have demonstrated the critical role these genes play in regulating skeletal muscle mitochondrial biogenesis. Studies have shown that deletion of PGC-1 α in skeletal muscle results in reduced oxidative capacity [48] and is critical for exercise-induced mitochondrial biogenesis [2, 49]. Using C2C12 cells, Liesa and colleagues [46] reported a $\approx 59\%$ increase in mitochondrial size with normal cristae morphology when these cells were transduced with PGC-1 β compared to controls. The authors [46] also found that electron transport chain protein complexes such as CcO were reduced by 25% in the gastrocnemius muscle of PGC-1 β null mice. In a glycolytic muscle-specific PGC-1 β overexpressing mouse model, Arany et al. [47] found that glycolytic muscles (i.e., quadriceps femoris) in these mice had significantly higher oxidative capacity (5-fold increase) than wild-type littermates [47]. Furthermore, they found that the tibialis anterior muscle, which is mainly glycolytic, was now predominantly oxidative in PGC-1 β overexpressing mice. In addition, microarray analysis revealed that many of the genes critical for oxidative phosphorylation were up-regulated in transgenic mice compared to controls [47]. The results of the above studies, therefore, suggest that specific genes (PGC-1 α and PGC-1 β) are responsible for inducing mitochondrial biogenesis and oxidative phosphorylation in predominantly glycolytic muscles.

In the current study, we also examined protein expression of Tfam, which previously has been shown to increase with exercise, (–)-epicatechin treatment, or both [10]. Consistent with Nogueira et al. [10] we also observed a significant increase in Tfam with (–)-epicatechin treatment which was maintained after cessation of the compound. We also used electron microscopy to determine potential differences in the ultrastructure of the plantaris muscle such as mitochondrial volume and cristae abundance. Mitochondrial volume increased, however, not statistically significant ($p = 0.056$) with (–)-epicatechin treatment. For cristae abundance, however, we found $\approx 34\%$ increase for the (–)-epicatechin-treated group compared to control. Furthermore, cristae abundance was maintained after cessation of (–)-epicatechin. St-Pierre et al. [50] reported that muscle cells expressing PGC-1 α or PGC-1 β had increased mitochondrial volume density and cristae abundance. The results of the current investigation indicate that (–)-epicatechin-induced mitochondrial biogenesis may be through a combination of pathways leading to mitochondrial proliferation in glycolytic muscle.

CcO is the terminal and rate-limiting enzyme of the ETC in intact cells [51–54]. CcO contains 13 polypeptide subunits where subunits I through III are encoded in the mitochondrial genome and the ten smaller subunits are encoded in the nucleus [55]. Notably, CcO subunits I and II are the catalytic subunits [56]. Specifically, CcO subunit II accepts electrons from cytochrome *c* which are then transferred to CcO subunit I eventually transferring them to molecular oxygen which is then reduced to water [55]. Few rodent and human exercise physiology studies have focused on changes to CcO subunits in skeletal muscle following endurance training.

In our previous paper on (–)-epicatechin, we showed an increase in CcO expression with endurance training and preservation of endurance training induced increases in CcO with (–)-epicatechin treatment [4]. Therefore, since CcO is a critical enzyme as part of the mitochondrial energy production process, we focused on this component of oxidative phosphorylation. (–)-Epicatechin treatment increased CcO levels by $\approx 70\%$ ($p < 0.05$) as assessed using an antibody against CcO subunit II. The increase in CcO with (–)-epicatechin treatment are similar to the results of Hüttemann et al. [4] who reported that (–)-epicatechin maintained endurance-training increases in CcO expression following 14 days of detraining in the quadriceps muscles of mice. Currently, no studies have examined mitochondrial respiration in skeletal muscle after acute exposure to (–)-epicatechin. We were, therefore,

interested in determining the effects of (–)-epicatechin on CcO specific activity after short-term (25 min incubation) *in vitro* incubation. Thus, we used the plantaris muscle of Sprague-Dawley rats (i.e., healthy muscle) that were the same age and gender as our LCR rats as well as the plantaris muscles of LCR rats in the control group. CcO specific activity was measured at maximal turnover (30 μ M substrate cytochrome *c*) after muscles were incubated with or without (–)-epicatechin for 25 min. Interestingly, we observed a significant increase in CcO specific activity for the healthy Sprague-Dawley muscle following (–)-epicatechin incubation, whereas muscles for the LCR rats did not show any increase in CcO specific activity. These results suggest that acute exposure to (–)-epicatechin cannot increase oxygen uptake in the muscle of LCR rats *in vitro*, but chronic exposure (30 days) to (–)-epicatechin does result in significant increases in CcO expression *in vivo*. Future studies will explore possible posttranslational differences on CcO between control and LCR rats that may reveal mechanistic insights into changes in cell signaling at the level of the mitochondrial oxidative phosphorylation system. This may reveal signaling deficits in the LCR rats that might, in part, explain their exercise intolerance.

Although the findings of the current study suggest that (–)-epicatechin enhances skeletal muscle angiogenesis and mitochondrial proliferation there are some potential limitations that need to be addressed in the future. First, the inclusion of a 'wild-type' control group or the HCR rats, although changing the research question we intended to answer here, would be interesting as a follow-up study to reveal the possible effect, both long- and short-term, on the indices reported in this study. The aim of the current study, however, was to determine if (–)-epicatechin could improve angiogenesis and mitochondrial proliferation in glycolytic muscle of a rodent model with congenital muscle dysfunction and whether the response is retained after cessation of the (–)-epicatechin. Second, we examined only glycolytic muscle (i.e., plantaris) and not oxidative muscle (i.e., soleus). As mentioned above, we selected the plantaris muscle because others [30] reported no difference in mitochondrial content between the soleus muscles of HCR and LCR rats, suggesting that the glycolytic muscle is more adaptive to pharmacological and exercise challenge. Also, we did not quantitatively assess exercise capacity via treadmill testing in the three groups pre- and post-intervention. We were unsuccessful despite numerous attempts to get the LCR rats to run on the treadmill prior to the start of our intervention period at the Wayne State University laboratory. Based on our experience with exercise studies using rodent models [4, 5, 10, 22], the LCR rats became excessively stressed when placed on the treadmill and would not move off of the shock grid despite being shocked intermittently or when the shock grid was turned off. Similarly, when the rats were placed on the treadmill belt and then the treadmill turned on the rats would 'ride' the belt and then stand on the shock grid. Since there are no empirical studies examining the effects of the endocrine response to stress on (–)-epicatechin metabolism we elected not to perform any type of functional testing under those circumstances.

In summary, the present study examined the role of (–)-epicatechin on increasing skeletal muscle capillarity and oxidative phosphorylation in genetically heterogeneous rats bred for innate low running capacity. The increase in capillarity may be associated by changes in the interaction between VEGF-A, TSP-1, and CD47. Notably, we found that the potential mechanism of action for (–)-epicatechin-induced mitochondrial biogenesis, in glycolytic muscle, may be associated with the p38 MAPK signaling pathway which was associated with increased downstream activation of PGC-1 proteins. The results of the current investigation, in conjunction with the findings from our previous studies utilizing inbred mouse models [4, 10] provide evidence that in glycolytic muscles (–)-epicatechin can influence skeletal muscle capillarity and mitochondrial oxidative capacity which are two critical components of the oxygen transport and utilization pathways.

Acknowledgments

M.H.M. designed the research; S.L.B. and L.G.K. created the rat model; all authors contributed to collection, analysis, and interpretations of data and developing the manuscript. All authors approved the final version of the manuscript for publication. We acknowledge the expert care of the rat colony provided by Molly Kalahar and Lori Gilligan. The LCR and HCR model can be made available for collaborative study (contact: brittons@umich.edu or lgkoch@umich.edu).

Funding This work was supported by start-up funds (M.H.M.) from Wayne State University, funding from the National Institutes of Health (NIH) P41 RR004050 (GAP) and (GM089900, M.H.). We also are appreciative to Ray Scott and Dr. Mark Ellisman for their support. The LCR-HCR rat model system was funded by the National Center for Research Resources grant R24 RR017718 and is currently supported by the Office of Research Infrastructure Programs/OD grant ROD012098A (to L.G.K. and S.L.B.) from the National Institutes of Health. S.L.B. was also supported by National Institutes of Health grant RO1 DK077200.

ABBREVIATIONS

p38 MAPK	mitogen-activated protein kinase (MAPK)
PGC-1	peroxisome proliferator-activated receptor gamma co-activator 1 proteins
VEGF-A	vascular endothelial growth factor
TSP-1	thrombospondin-1
LCR	low running capacity
HCR	high running capacity
CcO	cytochrome <i>c</i> oxidase

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Clinical Perspectives

Recently, there is increasing evidence that dark chocolate has positive effects on cardiovascular health [57, 58]. In addition, a recent meta-analysis has shown that the main compound in dark chocolate, (–)-epicatechin, improves circulation [59]. Our laboratory has shown, that in models of healthy skeletal muscle, (–)-epicatechin increases angiogenic and mitochondrial proliferation independent of exercise [10] as well as attenuating the effects of detraining [4]. What remains unknown, however, is whether these effects of (–)-epicatechin can be replicated in a model of diseased muscle. Therefore, the LCR rats present a unique model of compromised muscle function that translates to human disease [60]. The results of the current investigation indicated that (–)-epicatechin is associated with increased skeletal muscle capillarity as well as mitochondria after 30 days of treatment. Furthermore, these improvements in the skeletal muscle were maintained 15 after the cessation of the compound. These results are clinically significant, because (–)-epicatechin may have potential applications with diseases that impair skeletal muscle oxygen transport and utilization.

Figure 1. Capillary indices for the plantaris muscle of LCR rats

Comparison (mean $\pm$ SEM) of N_{CAF} (panel A), C/F_i (panel B), CD (capillary density, panel C), and CFPE (capillary-to-fiber perimeter exchange, panel D) between the three groups (n = 3 per group). Note: $p < 0.05$; *significantly different from Post (-)-Epi 15d group; †significantly different from (-)-Epi 30d group; and **both groups significantly different from control.

Figure 2. Protein expression of VEGF-A, TSP-1 and CD47

Representative western blot results from the plantaris muscle of LCR rats. †Indicates different from control and Post (-)-Epi 15d groups, $p < 0.05$; * indicates different from control group (mean $\pm$ SEM; $n = 3-4$ per group).

Figure 3. Protein expression of p38 MAPK pathway

Representative western blots from the plantaris muscle of LCR rats. * and †, significantly different ($p < 0.05$) from control group (mean $\pm$ SEM; $n = 5-6$ per group).

Figure 4. Protein expression of key mitochondrial biogenesis regulators
Representative western blots from the plantaris muscle of LCR rats. * and †, significantly different ($p < 0.05$) from control group (mean $\pm$ SEM; $n = 4$ per group).

Figure 5. Ultrastructure analysis of mitochondria in the plantaris muscle of LCR rats
Representative electron microscopy images for mitochondria (top panel) with arrows indicating cristae formation. The scale bar (third picture) represents 500 nm and is the same scale for all three photos. Bottom panel indicates analysis of mitochondrial volume density (A) and cristae abundance (B). *significantly different ($p < 0.05$) from control group; (mean $\pm$ SEM; n = 3–4 per group).

Figure 6. Protein expression of cytochrome *c* oxidase and specific activity in the plantaris muscle Representative western blot result of CcO II (A) and analysis (B) for the plantaris muscle. *significantly different ($p < 0.05$) from control group; (mean $\pm$ SEM; $n = 3$ per group). Panel C represents CcO specific activity of plantaris muscle of Sprague-Dawley rats (healthy muscle, HM) incubated in solution with or without (-)-Epicatechin at 20 μ M for 0 and 25 min. ($n = 5$ –6 experiments per group). *significantly different ($p < 0.05$) from control group; †significantly different ($p < 0.05$) from HM group at 25 min with no (-)-Epicatechin; and **significantly different ($p < 0.05$) from HM group at 25 min with and without (-)-Epicatechin incubation.

Table 1

Body and muscle masses of LCR rats (mean $\pm$ SEM).

	Groups		
	Control	(-)-Epi 30d	Post (-)-Epi 15d
	n = 7	n = 7	n = 7
Body mass (g)	474 $\pm$ 21	473 $\pm$ 21	499 $\pm$ 19
Plantaris (mg)	359 $\pm$ 24	381 $\pm$ 24	361 $\pm$ 23
Plantaris / body mass (mg·g ⁻¹)	0.76 $\pm$ 0.04	0.81 $\pm$ 0.04	0.72 $\pm$ 0.04

Note: No significant ($p > 0.05$) differences between groups.

Table 2Morphometric analysis of LCR rats (mean $\pm$ SEM).

	Groups		
	Control	(-)-Epi 30d	Post (-)-Epi 15d
<u>Plantaris Muscle</u>			
Area (μm^2)	3343 $\pm$ 65	3689 $\pm$ 82	3678 $\pm$ 123
Perimeter (μm)	246 $\pm$ 2	265 $\pm$ 3 ^a	273 $\pm$ 6 ^b
<i>R</i> ₉₅	44.6 $\pm$ 0.2	39.0 $\pm$ 0.6 ^c	42.4 $\pm$ 0.3 ^d

Note:

*R*₉₅ = estimated diffusion distance^a significantly ($p=0.03$) than control.^b significantly ($p=0.008$) than (-)-Epi 30d.^c significantly ($p<0.001$) than control^d significantly ($p=0.018$) than (-)-Epi 30d.