Original Paper

Acute Exercise Stimulates Carnitine Biosynthesis and OCTN2 Expression in Mouse Kidney

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Key Words

γ -butyrobetaine hydroxylase • Organic cation transporter-2 • carnitine • Peroxisome proliferator-activated receptor • Exercise

Abstract

Background/Aims: Carnitine is essential for the transport of long-chain FAs (FA) into the mitochondria for energy production. During acute exercise, the increased demand for FAs results in a state of free carnitine deficiency in plasma. The role of kidney in carnitine homeostasis after exercise is not known. **Methods:** Swiss Webster mice were sacrificed immediately after a 1-hour moderate intensity treadmill run, and at 4-hours and 8-hours into recovery. Non-exercising mice served as controls. Plasma was analyzed for carnitine using acetyltransferase and [14 C] acetyl-CoA. Kidney was removed for gene and protein expression of butyrobetaine hydroxylase (γ -BBH), organic cation transporter (OCTN2), and peroxisome proliferator-activated receptor (PPAR α), a regulator of fatty acid oxidation activated by FAs. **Results:** Acute exercise caused a decrease in plasma free carnitine levels. Rapid return of free carnitine to control levels during recovery was associated with increased γ -BBH expression. Both mRNA and protein levels of OCTN2 were detected in kidney after exercise and during recovery, suggesting renal transport mechanisms were stimulated. These changes were accompanied with a reciprocal increase in PPAR α protein expression. **Conclusions:** Our results show that the decrease in free carnitine after exercise rapidly activates carnitine biosynthesis and renal transport mechanism in kidney to establish carnitine homeostasis.

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Introduction

In mammals, fatty acid (FA) oxidation in tissues occurs in the mitochondria and is dependent on carnitine and the carnitine shuttle, an obligate system that transfers FAs from the cytosol into the mitochondria where they are oxidized for the production of energy [1-3]. During acute exercise, the demand for FAs is increased to support metabolic activity and evidence of a heightened role for carnitine is indicated by extraction of free carnitine from the plasma pool by tissues [4, 5]. Replenishment of plasma carnitine levels after exercise suggests that endogenous biosynthesis of carnitine is stimulated. The observation that reabsorption of carnitine is preserved during the postexercise recovery also indicates that renal uptake mechanisms are active in maintaining carnitine homeostasis [6].

The carnitine biosynthesis pathway has been extensively described in the literature and consists of a cascade of reactions involving methionine and lysine as precursors [7, 8]. Biosynthesis occurs in several tissues, and in the mouse, the main sources of carnitine are liver and kidney. In the final biosynthetic reaction, γ -butyrobetaine is hydroxylated by γ -butyrobetaine hydroxylase (BBH or butyrobetaine dioxygenase: E.C. 1.14.11.1) to produce carnitine, which is then released into the circulation and subsequently extracted by tissues by organic cation transporters (OCT). The sodium-dependent OCTN2 transporter, abundantly expressed in kidney, has a high affinity for carnitine and is considered the key transporter in renal reabsorption of carnitine from the urine [9]. Recent evidence indicates that carnitine biosynthesis in kidney is also dependent on systemic endothelial nitric oxide and endothelin-1 production [10].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone superfamily that regulate target gene expression by binding to specific response elements. PPAR α , one of the three isoforms identified to date, is expressed in tissues that exhibit an elevated capacity for FA oxidation, including heart, liver and kidney [11]. Therefore, release of non-esterified fatty acids (NEFAs) from adipose tissue, the natural ligand of this isoform, activates PPAR α expression resulting in the expression of genes involved in the activation and β -oxidation of FAs [12]. Ligand activation of PPAR α occurring with energy deprivation, such as during fasting and caloric restriction, also stimulates carnitine biosynthesis and uptake mechanisms into tissues to facilitate FA oxidation [13-15]. Recently, we have reported similar changes with chronic exercise training where BBH expression increased the carnitine concentration in plasma of rat [16].

Although many studies have examined the role of exercise training on carnitine homeostasis, the effects of a single exercise session on carnitine biosynthesis and uptake mechanisms in the mouse are not well-defined. In this study, we evaluated the effects of acute exercise on plasma carnitine levels as well as on the expression of BBH and OCTN2 in mouse kidney. We hypothesize that acute exercise, by decreasing free carnitine levels in the plasma, activates PPAR α resulting in increased expression of BBH and OCTN2 in kidney to restore carnitine homeostasis during the recovery period.

Materials and Methods

Animals and exercise protocol

Male Swiss Webster Mice (Charles River Laboratories, Wilmington, MA), 6-7 weeks of age, were used for this study. This strain of mice was used based on its ability to run without reluctance. After a one-week period of acclimatization, mice were subjected to 60 minutes of treadmill running at a speed of 19 meters/min, corresponding to $\sim 80\%$ $\text{VO}_{2\text{max}}$ [17]. Mice were euthanized immediately after exercise (acute group), 4 hours post exercise (4-hour group), and 8 hours post exercise (8-hour group). Non-exercising mice (control group) were placed on a stopped treadmill and were euthanized at the same time points as exercising mice. Mice were housed 4 per cage, with a 12:12 hour light-dark cycle and given food and water *ad libitum*. After exercise, mice were asphyxiated in an atmosphere of 100% CO_2 . The Midwestern University Institutional Animal Care and Use Committee approved this study, and animals used were cared for in accordance with the recommendations in The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996).

Tissue harvest and measurement of plasma metabolites

Blood was collected by cardiac puncture and transferred into chilled tubes. The blood was then centrifuged and the plasma was stored at -80°C. Plasma non-esterified fatty acids (NEFA) was measured using a commercially available kit (Wako Chemicals USA, Richmond, VA). Carnitine in plasma was measured by a radioenzymatic assay using carnitine acetyltransferase and [¹⁴C] acetyl-CoA [18]. Kidneys were removed, freeze snapped with tongs cooled to the temperature of liquid N₂, and stored for gene and protein expression.

Quantitative real-time PCR analysis

RNA isolation and PCR methods have been previously described [19]. Total RNA content was isolated from kidney using RNA stat-60 following the protocol provided by the manufacturer (AMS Biotechnology, Abingdon, UK). First-strand cDNA was synthesized using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using primers listed in Table 1 with 18S rRNA (Sigma Aldrich Life Science, The Woodlands, TX) used as the internal control. Relative mRNA levels were quantified using the delta-delta CT method.

Western blot analysis

Analysis of protein content in kidney was performed using methods previously described [16]. Samples (20 µg) were subjected to immunoblot analysis with the use of antibodies against PPAR-α, BBH, and OCTN2 (Santa Cruz Biotechnology Inc. Dallas, TX). As an internal control, blots were reprobed with an anti-actin antibody (Sigma Aldrich Life Science, The Woodlands, TX). Values in the control group were normalized to arbitrary units to make comparisons with the exercise groups.

Statistical Analysis

All values are reported as the mean ± S.E.M. Group mean differences were determined using a one-way analysis of variance, followed by a Tukey-Kramer comparison for post hoc analysis. A value of P < 0.05 was considered statistically significant.

Results

The effects of acute exercise and recovery on plasma NEFA levels are shown in figure 1. Sixty minutes of exercise resulted in a significant decrease in plasma NEFAs. NEFA levels during recovery showed a return towards control resting levels (4-hour group) and increased significantly at 8 hours of recovery (8-hour group) compared to the control group.

Plasma carnitine fractions are shown in table 2. Plasma free carnitine decreased below resting control levels during exercise (acute group) and underwent a progressive elevation during the recovery period

Table 1. Real-time polymerase chain reaction primers

Gene Product	Primer Sequences
BBH	AGCAGGACTCCAAGGAGAAT CACAAAGGTGGAGGAGAGAA
OCTN2	GGGCTACTTCGGACTTTCTC GGGAAGAACAAGGTGAGGAT
CPT-1a	CCAGTCTCAGCCTCTACGGC CACAGTGTCTGTCTCCGTG
PGC-1β	TGGAAAGCCCCTGTGAGAGT TTGTATGGAGGTGTGGTGGG
18S rRNA	AGTCCCTGCCCTTTGTACACA CGATCCGAGGGCCTCACTA

BBH, γ-butyrobetaine hydroxylase; OCTN, organic cation transporter. CPT-1a, carnitine palmitoyltransferase isoform 1a; PGC1-β, peroxisome proliferator-activated receptor-γ coactivator-1 beta.

Fig. 1. Effects of acute exercise on plasma non-esterified fatty acids. Values are reported as mean ± S.E.M. for 9-10 mice in each group. *, P < 0.05 compared with control; †, compared with acute exercise; ‡, compared with 4-hours.

(8-hour group). Plasma esterified carnitine rose in the acute exercise group but did not reach significant elevations above control levels. Recovery (4-, 8-hour groups) was marked by significant decreases in esterified carnitine and return to control levels.

Despite the changes in free and esterified carnitine, total carnitine in plasma remained unchanged after exercise and throughout recovery. However, the esterified-to-free ratio was increased in the acute group compared to the control group.

To determine if acute exercise stimulates carnitine biosynthesis, gene and protein expression of BBH were measured in kidney. As illustrated in figure 2, mRNA expression in kidney was increased in the acute group and remained elevated during the recovery period. This increase was not met by concomitant changes in protein expression, although expression of BBH was increased by ~35% and 50% in the acute group and recovery groups, respectively.

Carnitine is actively taken up by the kidney through transporter carriers. Figure 3 shows that exercise increased mRNA expression of the OCTN2 transporter and expression remained elevated 4 hours into recovery. A similar profile for OCTN2 protein expression was observed immediately after exercise and during recovery. In the acute group, protein expression of OCTN2 was increased nearly 2.5-fold and a significant elevation was observed 4 hours into recovery.

Recent evidence indicates that fasting activates PPAR α expression, which in turn regulates BBH and OCTN2 expression. The effect of one exercise session, however, on PPAR α expression is unclear. As shown in figure 4, protein levels of PPAR α were increased in kidney following exercise and remained elevated during recovery compared to the control group. To determine whether fatty acid oxidation was stimulated

Table 2. The effect of acute exercise on plasma carnitine levels

Group	Total carnitine	Free carnitine	Esterified carnitine	Esterified/Free Ratio
Control	88.6 $\pm$ 5.8	35.3 $\pm$ 3.1	53.5 $\pm$ 3.4	1.54 $\pm$ 0.10
Acute	81.0 $\pm$ 7.1	18.8 $\pm$ 2.1*	62.3 $\pm$ 5.8	3.42 $\pm$ 0.27*
4-hours	73.4 $\pm$ 6.4	30.5 $\pm$ 2.0†	42.9 $\pm$ 5.0†	1.40 $\pm$ 0.11†
8-hours	75.0 $\pm$ 2.1	32.6 $\pm$ 2.0†	42.8 $\pm$ 1.4†	1.36 $\pm$ 0.06†

Carnitine concentrations are expressed in μ Moles/L. Values are reported as mean $\pm$ SEM for 6-7 mice per group. * $P < 0.05$ vs control group; † $P < 0.05$ vs acute group.

Fig. 2. Effects of acute exercise on (A) mRNA expression (normalized for 18S) and (B) protein expression (normalized for actin) of BBH in mouse kidney. Kidney was harvested from mice immediately after exercise (acute) and 4 hours and 8 hours into recovery. Values are reported as mean $\pm$ S.E.M. for 8-9 mice in each group. *, $P < 0.05$ compared with control. BBH, γ -butyrobetaine hydroxylase.

by acute exercise, expression of the PPAR α mitochondrial target gene carnitine palmitoyltransferase-1a (CPT-1a) was determined. As illustrated in figure 5, expression of CPT-1a was increased by ~50%, although non-significantly, at 4 hours into recovery. However, gene expression of peroxisome proliferator-activated receptor- γ coactivator 1 (PGC-1 β) was significantly increased, indicating that mitochondrial respiratory function was stimulated by the exercise session [20].

Discussion

Caloric restriction, fasting, and exercise training increase the concentration of carnitine in plasma and tissues to enhance the oxidation of FAs, since carnitine is an essential cofactor in the transfer of this substrate into the mitochondria where β -oxidation occurs [14-16]. Under these conditions, the increase in plasma carnitine is mediated by the activation of PPAR α with subsequent stimulation of γ -BBH and OCTN2-mediated uptake mechanisms present in tissues [14, 15]. The reduction in plasma and hepatic carnitine levels induced by the metabolic stress of chronic high-fat feeding can be prevented with exercise training, indicating that exercise is a robust stimulus in reversing impairments in carnitine status [21]. Since acute exercise is also associated with an increased

demand for FAs and carnitine, it seems logical that carnitine biosynthesis and OCTN2 expression are stimulated in response to this stimulus, especially when exercise extracts free carnitine from plasma to support metabolism in working tissues [6]. Further, it is unknown whether this occurs in response to a single bout of exercise when the release of adipose tissue-derived NEFAs, the natural ligand for PPAR α , can potentially activate these homeostatic mechanisms. For these reasons, the present study was designed to determine whether the state of acute free carnitine deficiency induced by exercise stimulated carnitine homeostatic adjustments. Our results show that a single exercise session causes a dramatic decrease in the plasma levels of free carnitine in mouse, likely reflecting uptake of this cofactor into tissues. The rapid return of free carnitine in plasma to control levels during the recovery period suggests that carnitine biosynthesis was stimulated. Indeed, the observation that expression of γ -BBH was increased in kidney of exercising mice suggests that the rise in free carnitine in plasma is consistent with carnitine biosynthesis. Further, because increased protein levels of OCTN2

Fig. 3. Effects of acute exercise on (A) mRNA expression (normalized for 18S) and (B) protein expression of OCTN2 (normalized for actin) in mouse kidney. Kidney was harvested from mice immediately after exercise (acute) and 4 hours and 8 hours into recovery. Values are reported as mean $\pm$ S.E.M. for 8-10 mice in each group. *, $P < 0.05$ compared with control. OCTN2, organic cation transporter-2.

were detected in kidney after exercise, and considering the critical role of these transporters in the reabsorption of carnitine from urine by renal tubules [22], it is also likely that the increase in carnitine was attributed expression of this transporter.

The mechanism involved in eliciting the increase in carnitine biosynthesis and OCTN2 expression in kidney with acute exercise is unknown, although earlier studies in the 24-hour-fasted pig and with clofibrate treatment clearly support the role of PPAR α activation [13, 14]. The dependence of PPAR α on OCTN2 expression was confirmed in rat hepatocytes treated with the PPAR α antagonism (MK886), in cells over-expressing PPAR α as well as in PPAR α -null mice [22]. This transcription factor, activated by NEFAs from adipose tissue during acute exercise, could stimulate the synthesis of genes supporting FA oxidation, including those relating to carnitine biosynthesis and uptake [23, 24]. Indeed, although NEFA levels were actually decreased, indicating increased extraction for metabolic use, expression of the PPAR α target gene CPT-1a was increased by nearly 50% after exercise.

Fig. 5. Effects of acute exercise on mRNA expression (normalized for 18S) of (A) CPT-1a and (B) PGC-1beta in mouse kidney. Kidney was harvested from mice immediately after exercise (acute) and 4 hours and 8 hours into recovery. Values are reported as mean $\pm$ S.E.M. for 4 mice in each group. *, $P < 0.05$ compared with control; †, compared with acute exercise. CPT-1a, carnitine palmitoyltransferase isoform 1a; PGC1-beta, peroxisome proliferator-activated receptor- γ coactivator 1.

Fig. 4. Effects of exercise on PPAR- α protein (normalized for actin) expression in kidney. Kidney was harvested from mice immediately after exercise (acute) and 4 hours and 8 hours into recovery. Values are reported as mean $\pm$ S.E.M. for 8-10 mice in each group. *, $P < 0.05$ compared with control. PPAR, peroxisome proliferator-activated receptor- α .

Also noted was an increase in PGC-1 β mRNA expression, suggesting that mitochondrial respiration was increased by the stimulus of acute exercise [20]. Elevated use of FAs is consistent with the increased levels of esterified carnitine detected at the end of the exercise session and reported in earlier studies [5, 6, 25]. Increased esterification of carnitine reflects fatty acid oxidation by liver [25], induced through elevation in the glucagon-to-insulin ratio normally seen with acute exercise [26]. Despite this decrease in plasma NEFAs, lipolysis was enhanced as indicated by the markedly elevated levels of this substrate during the post-exercise recovery period. It is possible that the increase in PPAR α activation, which persisted for 8 hours after exercise, may also be involved in the disposal of the elevated levels of FAs in plasma during recovery, although further work is needed to confirm this effect.

Conclusion

We examined the effects of acute exercise on plasma free carnitine, carnitine biosynthesis and OCTN2 expression in kidney and found that carnitine homeostasis proceeded rapidly after exercise. Within 8 hours after exercise, the decrease in plasma free carnitine content was restored to non-exercising control levels and this was associated with increases in both BBH and OCTN2 expression in kidney. Our results are the first to highlight the role of the kidney in carnitine biosynthesis and homeostasis with exercise and we believe this might have important implications for the treatment of metabolic diseases. Stimulation of carnitine biosynthesis and uptake mechanisms by regular exercise could benefit kidney function, prevent the occurrence of renal calculi [27], and protect against renal injury [28].

Disclosure Statement

The authors declare that they have no competing interest.

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