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What is the Key Step in Muscle Fatty Acid Oxidation after Change of Plasma Free Fatty Acids Level in Rats?

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The purpose of this study was to discern the critical point in skeletal muscle fatty acid oxidation by changing plasma free fatty acids (FFA) level in rat. In the study, 3 key steps in lipid oxidation were examined after changing plasma FFA level by acipimox. The rates of both palmitate and palmitoyl-carnitine oxidation were decreased by decrease of plasma FFA level, however, carnitine palmitoyl transferase (CPT) 1 activity was not changed, suggesting CPT1 activity may not be involved in the fatty acid oxidation at the early phase of plasma FFA change. In the fasted rats, β-hydroxy acyl-CoA dehydrogenase (β-HAD) activity was depressed to a similar extent as palmitate oxidation by a decrease of plasma FFA level. This suggested that β-oxidation might be an important process to regulate fatty acid oxidation at the early period of plasma FFA change. Citrate synthase activity was not altered by the change of plasma FFA level. In conclusion, the critical step in fatty acids oxidation of skeletal muscles by the change of plasma FFA level by acipimox in fasting rats might be the β-oxidation step rather than CPT1 and TCA cycle pathways.

Key Words: Fatty acids oxidation, Skeletal muscle, β-oxidation, Acipimox

INTRODUCTION

Under physiological conditions, glucose and fatty acids are the main substrates for energy generation in the skeletal muscle. As whole-body metabolism changes, the contribution of glucose and fatty acids to the energy production in skeletal muscles alters as well. During fasting or diabetes, for example, the contribution of fatty acids to muscle energy production increases, whereas the utilization of glucose is reduced via increased circulating fatty acid concentrations and the enhanced supply of these substrates to the muscle. Both the liver and skeletal muscle can oxidize fatty acids, but the skeletal muscle plays more important role in whole body lipid oxidation, because of its greater relative mass. At rest, the lipid oxidation contributes significantly to the overall energy needs. Skeletal muscle accounts for 20% of the basal metabolic rate, and up to 90% of the energy requirements of resting muscle are obtained from lipid oxidation (Dagenais et al, 1976). Lipids play many structural and metabolic roles and dietary fat has great impact on metabolism and health. In many tissues, even under fed conditions, fatty acids are oxidized preferably to glucose, but particularly under the condition of caloric deficit or starvation to spare glucose for the brain and erythrocytes. There are several key steps to oxidize plasma free fatty acids (FFA) in the skeletal muscle. First, FFA must enter mitochondrial matrix across outer and inner mitochondrial membranes. Next, oxidation of long chain fatty acid (LCFA) begins with activation of the fatty acid in both sides of mitochondrial membranes. Active fatty acids formed outside mitochondria cross the mitochondrial membrane by a process that requires carnitine. Carnitine palmitoyl transferase (CPT) 1 in the outer mitochondrial membrane is essential to oxidize LCFA, because LCFA can not enter the mitochondria without CPT1. Therefore, CPT1 is believed to play a key role in the regulation of mitochondrial β-oxidation of fatty acids in all human tissues (McGarry & Brown, 1997) and control a critical point in cellular lipid metabolism. Acyl-CoA in mitochondrial matrix derived from plasma free fatty acids has to undergo β-oxidation and is then finally oxidized through TCA (tricarboxylic acid) cycle. Therefore, β-oxidation is also an important step to enter TCA cycle, and also mitochondrial oxidative capacity is responsible for oxidation in TCA cycle. Based on these points, the study was conducted to investigate what is the more important step in skeletal muscle fatty acid oxidation, when the plasma FFA level was changed by acipimox (5-methylpyrazine carboxylic acid-4-oxide), which has lipid-lowering property by inhibition of hormone-sensitive lipase in hyperlipidemia patients (Tornvall & Walldius, 1991).

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ABBREVIATIONS: FFA, free fatty acids; CPT1, carnitine-palmitoyl transferase 1; β-HAD, β-hydroxy acyl-CoA dehydrogenase; TCA cycle, tricarboxylic acid cycle.

METHODS

Animal experiments

Male Sprague-Dawley rats, weighing approximately 250 g, were used for determination of fatty acid oxidation of white and red skeletal muscles. Rats were injected with saline or acipimox. Before sacrifice, a mixture containing 90 mg/ml ketamine and 10 mg/ml xylazine at 0.1 ml/100 g body weight was administered, and the quadriceps muscle was quickly removed and trimmed of any connective tissue and fats.

Groups and acipimox

Twenty seven rats were divided to fed (n=9), fasted (n=9) and fasted-acipimox (n=9) groups. Rats were subcutaneously injected with acipimox (50 mg/kg) to reduce plasma free fatty acids level (Donati et al, 2004) at 2 hrs intervals for 12 hrs before the experiment.

Preparation of muscle homogenate

Tissue samples were blotted, weighed, and placed in 10 volumes of a homogenization buffer containing 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, and 2 mM ATP, pH 7.4. Samples were thoroughly minced with surgical scissors and homogenization buffer was added to yield 20-fold diluted (wt:vol) homogenate and the whole sample was transferred to 3 ml Potter-Elvehjem glass homogenizer. Muscle suspensions were homogenized on ice with a Teflon pestle at 10 passes across 30 sec at 1,200 rpm. This method was found to yield intact mitochondria for metabolic studies (Scholte et al, 1997; Kim et al, 2002a).

Rate of fatty acid oxidation

The oxidation rate of fatty acid in tissue homogenates was measured by counting $^{14}CO_2$ produced from palmitic acid or palmitoyl-carnitine (Kim et al, 2000). Briefly, 40 μl of 20-fold diluted tissue homogenates were preincubated with 5% CO_2-95% O_2 mixture at 30°C for 15 min, and 160 μl of reaction mixture (pH 7.4) was then added to the above preincubated tissue homogenates. The final incubation mixture contained 100 mM sucrose, 10 mM Tris-HCl, 5 mM potassium phosphate, 80 mM potassium chloride, 1 mM magnesium chloride, 2 mM L-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM coenzyme A, 1 mM dithiothreitol, 0.2 mM EDTA, and 0.3% bovine serum albumin (BSA), and 0.2 mM palmitate-1-^{14}C or ^{14}C-palmitoyl-carnitine (0.5 uCi) as substrates. After 60 min of incubation at 30°C, 100 μl of 4N sulfuric acid was added to stop the reaction. $^{14}CO_2$ produced during the 60 min incubation was trapped with 200 μl of 2M sodium hydroxide, and trapped $^{14}CO_2$ was determined by liquid scintillation counting by the use of 5 ml liquid scintillation cocktail.

CPT1 activity

CPT1 activity in tissues homogenates was determined. CPT1 activity in the selected tissues was measured using modification of the method (Kim et al, 2000) developed by McGarry et al. (1983) and Zierz & Engel (1987). This method measures the rate of palmitoylcarnitine formation from palmitoyl-CoA and carnitine. Ten μl of 20-fold diluted tissue homogenate was preincubated at 30°C in a microcentrifuge tube for 10 min. Reaction was initiated by adding 90 μl of the following reaction mixture to tissue homogenates preincubated at 30°C for 10 min. The incubation mixture (pH 7.4) included 117 mM Tris-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM $MgCl_2$, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.1% BSA, 50 uM palmitoyl CoA and 0.2 mM ^{3}H-carnitine (0.5 uCi) as the substrate. The reaction was terminated with 60 μl of 1.2 mM ice-cold HCl. The ^{3}H-palmitoylcarnitine formed was extracted with water-saturated butanol and determined by liquid scintillation counting.

Citrate synthase and β-hydroxy acyl-CoA dehydrogenase (β-HAD) activities

Citrate synthase activity as a marker enzyme for TCA cycle (Srele PA, 1969), and β-HAD activity as a key enzyme for β-oxidation (Gollnick & Saltin, 1982) were measured.

Analytical procedure

Concentration of plasma free fatty acids from the abdominal artery was determined using enzymatic method.

Statistical analysis

Values are expressed as means±SE. The significance of difference between two groups was evaluated using Student's t-test. For multiple comparisons, a one-way analysis of variance (ANOVA) was used. When ANOVA showed significant differences, post-hoc analysis was performed with the Newman-Keuls multiple range test by SPSS.

RESULTS

This study was conducted to investigate whether the regulation mechanism of skeletal muscle fatty acid oxidation was the rate limiting steps by changing plasma free fatty acids level as previously reported, using acipimox. As shown in Table 1, plasma free fatty acid concentration (μM) was increased by fasting (676±37), compared with fed (224±10), and acipimox (364±42) decreased it by ~47%, compared with fasted group. The rate of palmitate oxidation (nmole/g/min) in white skeletal muscle was decreased by acipimox injection (0.95±0.11) compared with saline group (1.36±0.15), and the oxidation rate of palmitate in red skeletal muscle was decreased by acipimox injection (7.41±0.49), compared with saline group (9.48±0.72) (Fig. 1). Acid-soluble material (nmole/g/min) resulting from palmitate oxidation in white skeletal muscle was also decreased by acipimox injection (19.5±2.0), compared with saline group (27.3±2.2), and the oxidation rate of palmitate in red skel-

Table 1. Plasma FFA concentration in acipimox treated rats

	Fed	Fasted	Fasted-ACP
Plasma free fatty acids (μmole)	224±10	676±37**	363±42*,#

Values are mean SE from 9 rats. ACP; acipimox, FFA; free fatty acids. *p<0.05 vs. fed, **p<0.01 vs. fed, #p<0.01 vs. fasted.

etal muscle was decreased by acipimox injection (64.8±4.9) compared with saline group (80.5±6.2) (Fig. 2). As shown in Fig. 3, the oxidation rate of palmitoyl-carnitine (nmole/g/min) of white skeletal muscle was decreased by acipimox injection (0.87±0.06), compared with saline group (1.24±0.15), and the oxidation rate of palmitoyl-carnitine in red skeletal muscle was decreased by acipimox injection (4.52±0.19), compared with saline group (6.03±0.33). As shown in Fig. 4, CPT 1 activity was not different between acipimox and saline groups in white (28.0±3.8 and 29.3±4.2 in saline and acipimox injected rats, respectively) and red skeletal muscles (58.5±3.3 and 59.3±3.5 in saline and acipimox injected rats, respectively). As shown in Fig. 5, citrate synthase activity was not different between saline and

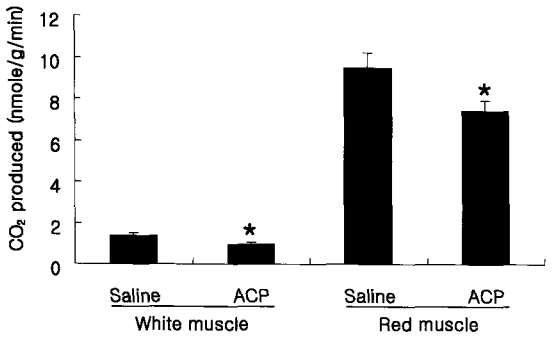

Fig. 1. Oxidation rate of palmitate in white and red skeletal muscles. ACP; acipimox. Values are mean±SE. *p<0.05 vs. saline.

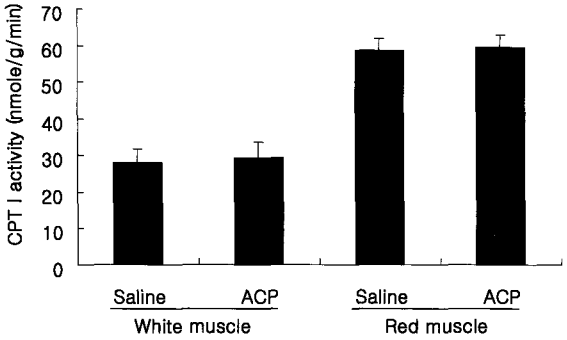

Fig. 4. Carnitine palmitoyl transferase 1 activity in white and red skeletal muscles in acipimox-treated fasted rats. ACP; acipimox. Values are mean±SE.

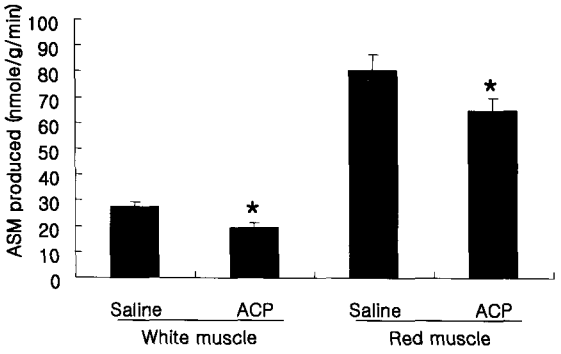

Fig. 2. Acid-soluble materials (ASM) drived from palmitic acid oxidation in white and red skeletal muscles in acipimox-treated fasted rats. ACP; acipimox. Values are mean±SE. *p<0.05 vs. saline.

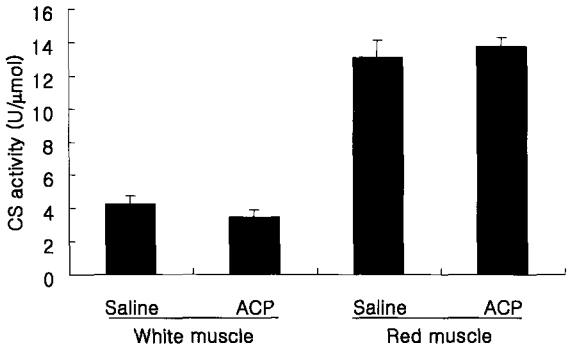

Fig. 5. Citrate synthase (CS) activity of white and red skeletal muscles in acipimox-treated fasted rats. ACP; acipimox. Values are mean±SE.

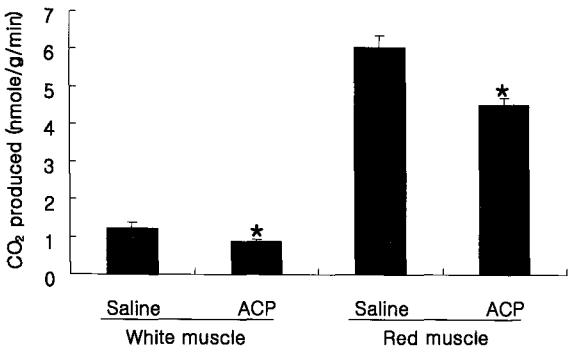

Fig. 3. Oxidation rate of palmitoyl-carnitine in white and red skeletal muscles in acipimox-treated fasted rats. ACP; acipimox, Values are mean±SE. *p<0.05 vs. saline.

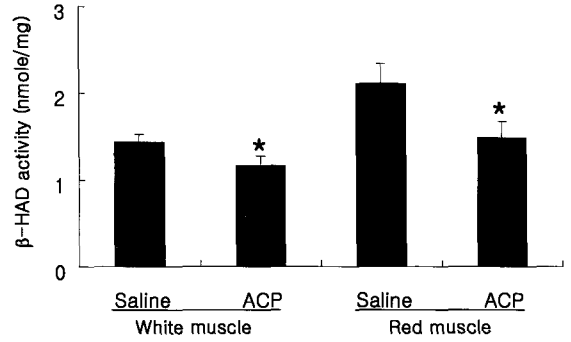

Fig. 6. β-hydroxy acyl-CoA dehydrogenase (β-HAD) activity in white and red skeletal muscles. ACP; acipimox. Values are mean±SE. *p<0.05 vs. saline.

acipimox groups in white (4.23±0.52 and 3.49±0.41 in saline and acipimox injected rats, respectively) and red skeletal muscles (13.10±1.02 and 13.77±0.51 in saline and acipimox injected rats, respectively). As shown in Fig. 6, β-HAD activity (mmole/mg) of white skeletal muscle was decreased by acipimox injection (1.16±0.11) compared with saline group (1.43±0.09), and the oxidation rate of palmitate (mmole/g/min) in red skeletal muscle was decreased by acipimox injection (1.48±0.19), compared with saline group (2.11±0.23).

DISCUSSION

The skeletal muscle plays an important role in whole-body lipid oxidation. During fasting (postabsorptive) condition, lipid oxidation is the predominant metabolic activity of resting skeletal muscle (Dagenais et al, 1976) with approximately 40% of the fatty acids supplied being cleared with one circuit through the skeletal muscle capillary system (van der Vusse and Reneman, 1996). At rest, the lipid oxidation contributes significantly to overall energy needs; up to 90% of the energy requirements of resting muscle are obtained from fatty acid oxidation (Dagenais et al, 1976; Bulow, 1988). Therefore, by virtue of its mass, the skeletal muscle is quantitatively a pivotal factor in terms of fate of circulating lipid.

Fasting or diabetes increases plasma free fatty acid level and facilitates more utilization of fat for energy production, whereas the utilization of glucose is reduced via increased circulating fatty acid concentrations and an enhanced supply of these substrates to the muscle. Decreased plasma fatty acid level in healthy men by acipimox (Watt et al, 2004) during exercise and lean NIDDM patients (Piatti et al, 1996) significantly decreases muscle lipid oxidation and non-oxidative glycolysis.

According to Randle's (Randle, 1998) glucose-fatty acid cycle, increased usage of free fatty acids as energy source can cause insulin resistance in peripheral tissues, however fasting or exercise improves insulin sensitivity, although it increases fatty acid oxidation. Nevertheless, its' precise mechanisms at key pathways of lipid oxidation are not yet known.

In the present study, 3 key steps in fatty acids oxidation in mitochondria were checked by changing plasma FFA level with acipimox. A regulatory step in the fatty acids oxidation was found to be the transfer of nonpermeable LCFA across mitochondrial membranes. This process is regulated by CPT1 (van der Vusse & Reneman, 1996): CPT1 in outer mitochondrial membrane is essential to oxidize LCFA, because LCFA can not enter the mitochondria without CPT1. Therefore, CPT1 is believed to play a key role in the regulation of mitochondrial oxidation of fatty acids in all human tissues (McGarry & Brown, 1997) and to control a critical point in cellular lipid metabolism.

Palmitic acid (palmitate) is a long chain fatty acid with 16 carbons and needs CPT1 to enter the mitochondria. Pamitoyl-carnitine is an acyl-CoA derivative of palmitic acid and does not require CPT1 to transport across mitochondrial membranes. The oxidation rates of both palmitate and palmitoyl-carnitine were decreased by decrease of plasma FFA level, and CPT1 activity was not changed. This suggested that CPT1 activity may not be involved in fatty acid oxidation during 12 hour of plasma FFA change. Another rate limiting step for fatty acids oxidation is β-oxidation, because acyl-CoA of fatty acids in mitochondrial matrix has to undergo β-oxidation. In the present study the activity of β-HAD, the key enzyme in β-oxidation, was depressed at same extent as palmitate oxidation by a decrease of plasma FFA level in fasted rats suggesting that β-oxidation might be an important factor to regulate fatty acid oxidation during 12 hour of plasma FFA change. Citrate synthase is involved in oxidative production of ATP and is found to be directly proportional to muscle mitochondrial content (Gollnick & Saltin, 1982; Howald et al, 1985). In the present study, citrate synthase activity was not altered by change of plasma FFA level. These findings together suggest that a decrement in muscle oxidative capacity by a decrease of plasma FFA level might be caused by a decrease in β-oxidation of fatty acid without changes at post-CPT1 pathways. In addition, there was no difference in muscle lipid oxidation at 3 key steps by changes of plasma FFA between types of muscles. This suggests that the regulatory mechanism of fatty acid oxidation in white and red skeletal muscles would be the same or similar.

In conclusion, the critical step in fatty acids oxidation in both red and white skeletal muscles after changes of plasma FFA level by acipimox in fasting rats appears to be the β-oxidation step rather than CPT1 and TCA cycle pathways.

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REFERENCES

Bulow J. Lipid mobilization and utilization. *Principles of Exercise Biochemistry*. 1st ed. Basel, Karger, p 140−163, 1988

Dagenais GR, Tancredi RG, Zierlier KL. Free fatty acid oxidation by forearm muscle at rest, and evidence for an intramuscular lipid pool in human forearm. *J Clin Invest* 58: 421−431, 1976

Donati A, Cavallini G, Carresi C, Gori Z, Parentini I, Bergamini E. Anti-aging effects of anti-lipolytic drugs. *Exp Gerontol* 39: 1061−1067, 2004

Gollnick PD, Saltin B. Significance of skeletal muscle oxidative enzyme enhancement with endurance training: hypothesis. *Clin Physiol* 2: 1−12, 1982

Howald H, Hoppeler H, Claasen H, Mathieu O, Straub R. Influence of endurance training on the ultrastructural composition of the different muscle fiber types in humans. *Pfluegers Arch* 403: 369−376, 1985

Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 279: E1039−E1044, 2000

Kim JY, Hickner RC, Dohm GL, Houmard JA. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. *Metabolism* 51: 460−464, 2002a

Kim JY, Koves TR, Yu GS, Gulick T, Cortright RN, Dohm GL, Muoio DM. Evidence of a malonyl-CoA-insensitive carnitine palmitoyltransferase I activity in red skeletal muscle. *Am J Physiol Endocrinol Metab* 282: E1014−E1022, 2002b

McGarry JD, Brown NF. The mitochndrial carnitine palmitoyltransferase system from concept to molecular analysis. *Eur J Biochem* 244: 1−14, 1997

McGarry JD, Mills SE, Long CS, Foster DW. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues

of the rat. *Biochem J* 214: 21−28, 1983
Piatti PM, Monti LD, Davis SN, Conti M, Brown MD, Pozza G, Alberti KG. Effects of an acute decrease in non-esterified fatty acid levels on muscle glucose utilization and forearm indirect calorimetry in lean NIDDM patients. *Diabetologia* 39: 103−112, 1996
Randle PJ. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14: 263−283, 1998
Scholte HR, Yu Y, Ross JD, Oosterkamp II, Boonman AM, Busch HF. Rapid isolation of muscle and heart mitochondria, the lability of oxidative phosphorylation and attempts to stabilize the process in vitro by taurine, carnitine and other compounds. *Mol Cell Biochem* 174: 61−66, 1997
Srele PA. Citrate synthase. *Meth Enzymol* 13: 3−26, 1969
Tornvall P, Walldius GW. A comparison between nicotinic acid and acipimox in hypertriglyceridaemia--effects on serum lipids, lipoproteins, glucose tolerance and tolerability. *J Intern Med* 230: 415−421, 1991
Van der Vusse GJ, Reneman RS. Lipid metabolism in muscle. In: Rowell LB, Shepherd JT ed, *Handbook of Physiology*. 1st ed. Oxford University Press, New York, p 952−994, 1996
Watt MJ, Holmes AG, Steinberg GR, Mesa JL, Kemp BE, Febbraio MA. Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E120−E127, 2004
Zierz S, Engel AG. Different sites of inhibition of carnitine palmitoyltransferase by malonyl-CoA, and by acetyl-CoA and CoA, in human skeletal muscle. *Biochem J* 245: 205−209, 1987