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Evaluation of Theophylline-Stimulated Changes in Carnitine Palmitoyltransferase Activity in Skeletal Muscle and Liver of Rats

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The effect of theophylline treatments on the activity of carnitine palmitoyltransferase (CPT) in skeletal muscle and the liver of rats was investigated. Theophylline was administered at 100 mg/kg bw/day and effects were monitored after a treatment period that lasted between a week and five weeks. Results showed that a significant increase in the activity of CPT was observed in skeletal muscle of theophylline-treated groups as compared to either control or placebo groups. However, there was no significant change in the activity of CPT in the hepatic tissues of theophylline-treated groups. The observed discrepancies in activity of CPT might be due to the presence of two isoenzymes, the muscle type (M-CPT) and liver type (L-CPT); it is possible that theophylline affects only M-CPT activity.

Keywords: Carnitine palmitoyltransferase, Lipolysis, Liver, Muscle, β-oxidation, Rat, Theophylline

INTRODUCTION

For the transport of long-chain fatty acid across the mitochondrial membrane, prior to undergoing β-oxidation in the mitochondrial matrix, carnitine palmitoyltransferases palmitoyl-CoA: carnitine o-palmitoyltransferase; (EC 2.3.1.21, CPT I and CPT II) and the carnitine acylcarnitine translocase are required.[1,2] CPT is widely distributed in animal tissues, and is present as a membrane-bound enzyme in mitochondria and peroxisomes.[1] CPT I, located on the outer mitochondrial membrane, catalyzes the carnitine-dependent esterification of palmitoyl-CoA to palmitoylcarnitine. Palmitoylcarnitine is then transported across the inner mitochondrial membrane in exchange for carnitine by carnitine acylcarnitine translocase. CPT II, located on the inner mitochondrial membrane, catalyzes a second esterification, generating palmitoyl-CoA and carnitine inside the mitochondrial matrix (Figure 1).[1,2] CPT I is thought to exert the more important regulator influence on rates of mitochondrial fatty acid β-oxidation.[1] A very important aspect of regulation of mitochondrial fatty acid β-oxidation by CPT I is its inhibition by malonyl-CoA, the first committed intermediate in fatty acid synthesis.[1] The activity and the

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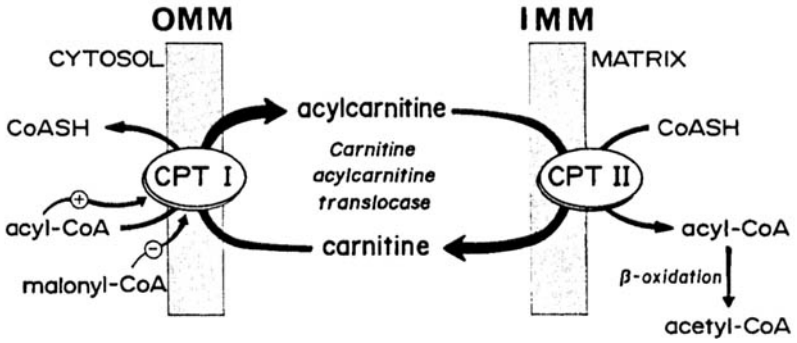

FIGURE 1 Carnitine-dependent transport of fatty acid acyl-CoA across inner mitochondrial membrane (IMM). Long-chain fatty acid acyl-CoA thioesters are converted to acylcarnitine esters by carnitine palmitoyltransferase I (CPT I), followed by transport through IMM by carnitine acylcarnitine translocase. CPT II reforms acyl-CoA thioesters in the matrix. Malonyl-CoA inhibits CPT I whereas palmitoyl-CoA activates it. OMM: outer mitochondrial membrane.

sensitivity to malonyl-CoA of the enzyme are regulated both on the short[3] and on the long term[4] by the nutritional and the hormonal status of the animal. Thus, hormones such as insulin,[5] glucagon[6] and oestrogens[7] change the properties of CPT I.

Inherited deficiency of CPT causes muscle weakness, cramps and myoglobinuria in adults[8] and coma, non-ketotic hypoglycemia, elevation of transaminases and free fatty acids in infants.[9]

Theophylline (1,3-dimethylxanthine) is used as a drug in the treatment of acute and chronic obstructive lung disease, in modern therapeutics and in the management of apnea of prematurity.[10] It is known for its narrow therapeutic range, interactions with other drugs and many side effects. Minor side effects tend to occur in some patients with plasma levels above 15 μg/mL and they are especially frequent with levels above 25 μg/mL (therapeutic range 10–20 μg/mL).[11] Major toxic complications are cardiac arrhythmias, hypotension and seizures and are often difficult to control. These toxic events can be lethal or lead to permanent neurological damage despite optimal supportive treatment and extracorporeal drug removal.[10] It is known to stimulate skeletal muscle, central nerve system respiratory centers and relax airway smooth muscle.[12] Theophylline causes an increased lipolysis in the adipose tissue and consequently enhances the levels of plasma free fatty acid.[13] Accumulation of cAMP levels following inhibition of phosphodiesterase, and antagonism of adenosine receptors have also been reported due to theophylline treatment.[14] Recently, we showed that theophylline administrations caused significantly increased in plasma levels of carnitine in rats.[15] Moreover, we observed that theophylline effects on the levels of carnitine in the skeletal muscle,[16] heart[17] but not in liver of rats.[16]

The object of the present study was to determine the effect of daily administration of theophylline on the activity of carnitine palmitoyltransferase in skeletal muscle and the liver of rats. To the best of our knowledge, no data are available on the effect of theophylline treatment on the activity of carnitine-dependent enzymes in any species.

MATERIALS AND METHODS

Chemicals

L-Carnitine hydrochloride, tris-(hydroxymethyl) aminomethane (Tris), 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid disodium salt (EDTA), carnitine acetyltrans-

ferase (CAT) and the lithium salt of acetylcoenzyme A, were purchased from Sigma Chemical Company (St Louis, MO, USA). Theophylline was obtained from Fluka Chemie AG (Buchs, Switzerland). Catalase (EC 1.11.1.6) was purchased from Winlab (Maidenhead, Berkshire, U.K). All other chemicals used were of analytical grade. Glass distilled water was used throughout.

Animal Care

A total of 150 male Wistar rats weighing between 200–265 g were obtained from the Breeding Laboratory, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. They were housed separately and fed water and rodent food *ad libitum*. They were subjected to a one-week acclimatization period in an air-conditioned room maintained at 21 °C with a relative humidity of 60%. A recurring cycle of 12 h each of light and darkness periods was maintained throughout the experimental period. At the beginning of the experiment, the animals were divided randomly into three groups, each comprising of 50 rats in properly suspended metabolic cages with stainless steel wire-mesh floor. Group 1 (control), 2 (placebo) and 3 (theophylline-treated) comprised of control without dosing, placebo receiving saline solution and treated receiving theophylline solution, respectively. An aqueous solution of theophylline (100 mg/kg bw/day) was used as described below.

Dosing Method

Normal saline solution or theophylline solution was dosed orally via the gastric lavage technique. Further details of the dosing method have been described previously.[15] After sacrifice, the extensor digitorum longus muscle from both hind limbs and the liver were removed quickly, weighed, frozen in liquid nitrogen and stored in a frozen condition at –70 °C until further processed. In all experiments, pair-fed control rats were used to assure that the effect was chemically-induced and not due to decreased food intake.

Sample Preparation

500 mg tissues were homogenized (10% w/v) at 4 °C in ice-cold 50 mM potassium phosphate buffer pH 7.5 containing 0.5 mM EDTA and 0.1% Triton X-100 using a stainless steel Omni-Mixer homogenizer (Omni International, Inc, Gainesville, VA, USA). CPT activity was measured after using multiple freeze-thaw cycles of the tissue homogenate.

Enzyme Assay

Total CPT activity was measured in the direction of palmitoyl-CoA formation. The colorimetric procedure described previously.[18] The assay is based on measuring the initial rate of total CoASH formation by the DTNB reaction from palmitoyl-CoA by tissue homogenate individually with carnitine and without carnitine. Cuvette A contained 100 mM Tris-HCl buffer, pH 7.8, 1.25 mM EDTA, 0.1 mM DTNB, 0.15 mM palmitoyl-CoA and 1.25 mM carnitine in a final volume of 1.0 mL to measure the total CoASH formed from palmitoyl-CoA by CPT plus all other competing reactions that produce CoASH such as acyl-CoA hydrolase, as well as any other reactions that produce reduced thiol groups. Cuvette B contained the identical substances in cuvette A except that 1.25 mM carnitine solution was omitted to measure the total CoASH formed by the competing reactions, the hydrolase assay, minus the CPT activity. The CPT activity was calculated as follows:

$$\begin{aligned}&\text{Total CoASH formed by CPT}\\&\quad = \text{Total CoASH formed in cuvette A}\\&\quad - \text{Total CoASH formed in cuvette B.}\end{aligned} \qquad (1)$$

The reaction was initiated by the addition of an aliquot of tissue homogenate, mixing

immediately and the rates were followed at 25 °C by monitoring the change in absorbance at 412 nm using a Ultrospec 2 000 UV/visible Spectrophotometer (Pharmcia Biotech Ltd, Science Park, Cambridge, England). The molar extinction coefficient (ϵ) of 13 600 M^{-1} cm^{-1} for 5′-thio-2-nitrobenzoate was used for the calculations. One unit of CPT activity is defined as the amount of enzyme catalyzing the release of 1 nmol CoASH/min/mg non-collagen protein (NCP) under the assay conditions.

Protein Determination

Non-collagen protein (NCP) was isolated by the procedure described by Lilienthal *et al.*[19] An aliquot of tissue homogenate was added to 50 mM NaOH and incubated at room temperature for 18 h. After centrifugation, the protein was then estimated by the modified Lowry method using bovine serum albumin as protein standard.[20]

Determination of Theophylline Levels

Theophylline was measured using a fluorescence polarization immunoassay method (Abbott TDx system, Abbott Laboratories, Wokingham, Berks, UK).

Statistical Analysis

Each sample was run in duplicate. The CPT activity was expressed as mean ± SD nmol/min/mg NCP for $n = 10$ rats per week. The changes in CPT activity were compared using one-way ANOVA analysis followed by Dunnett's test for multiple comparison test. Bartlett's test was used for homogeneity of variances. $P < 0.05$ was taken as the level of statistical significance. Statistical analysis was performed by means of GraphPad Prism® package for personal computers (GraphPad™ Software, Inc., San Diego, USA).

TABLE I Carnitine palmitoyltransferase (CPT) activity in skeletal muscle of control, placebo and theophylline-treated groups of rats

Time (Week)	CPT Activity (nmol/min/mg NCP)		
	Control	Placebo	Treated
0	0.79 ± 0.22		
1	0.77 ± 0.12	0.75 ± 0.11	1.66 ± 0.08†
2	0.74 ± 0.11	0.78 ± 0.12	1.45 ± 0.11†
3	0.81 ± 0.23	0.76 ± 0.10	1.76 ± 0.10†
4	0.79 ± 0.19	0.80 ± 0.22	1.89 ± 0.16†
5	0.77 ± 0.16	0.73 ± 0.17	1.69 ± 0.12†

Data are expressed as means ± SD, $n = 10$ rats/week. NCP: non-collagen protein. †Significantly different as compared to either control or placebo group ($P < 0.01$, Dunnett's multiple comparison test).

RESULTS

The effects of orally administrated theophylline (100 mg/kg bw/day) on the activity of CPT in skeletal muscle and liver were evaluated in adult male rats for five-week interval treatments. Table I shows the means (± SD) of carnitine palmitoyltransferase (CPT) activity in skeletal muscle of control, placebo and theophylline-treated rats. No significant difference in CPT activity was observed in placebo groups as compared to control groups. However, the theophylline-treated groups showed a significantly high CPT activity as compared to either control or placebo groups ($P < 0.01$).

Table II shows the means (± SD) of CPT activity in the liver of control, placebo and

TABLE II Carnitine palmitoyltransferase (CPT) activity in liver of control, placebo and theophylline-treated groups of rats

Time (Week)	CPT Activity (nmol/min/mg NCP)		
	Control	Placebo	Treated
0	9.87 ± 1.22		
1	9.18 ± 1.51	10.11 ± 1.22	9.77 ± 1.21†
2	11.01 ± 1.22	8.12 ± 1.31	8.61 ± 1.23†
3	8.98 ± 1.33	9.11 ± 1.21	9.21 ± 1.13†
4	10.13 ± 1.44	8.79 ± 0.97	10.09 ± 1.11†
5	9.45 ± 1.23	9.08 ± 1.42	10.16 ± 0.77†

Data are expressed as means ± SD, $n = 10$ rats/week. NCP: non-collagen protein. †Not significant as compared to either control or placebo group (Dunnett's multiple comparison test).

theophylline-treated rats for five-week interval treatments. No significant difference in CPT activity was observed in the theophylline-treated groups as compared to either placebo or control groups.

DISCUSSION

The efficacy of theophylline as a metabolic-inducer agent by increasing plasma levels of free fatty acids is well known.[14] CPT facilitates transfer of long-chain acyl groups across the inner mitochondrial membrane which helps in maintaining acyl-CoA/CoASH homeostasis across cellular organelles, especially in the mitochondria.[1] In this study we investigated the effect of theophylline feeding on the activity of CPT in the liver and skeletal muscle of rats for five-week interval treatments.

In the present study, a dose of 100 mg/kg bw/day was administered orally which resulted in a mean ± SD plasma theophylline concentration of $15.9 \pm 0.7\,\mu g/mL$.[17] This concentration is within the safe therapeutic range employed for humans (5–20 μg/mL).[13] At this dose, theophylline has been employed in bronchodilation in acute bouts of asthma and in the management and prevention of neonatal apnea.[13]

Previously we observed a body weight gain in theophylline-treated rats, however, the mean absolute heart, kidneys and liver weights were not significantly different from either control or placebo groups.[16,17,21] These changes were, therefore, considered as related to treatment with theophylline. Moreover, we reported that theophylline treatment caused an increase in food intake in theophylline-treated rats compared to either control or placebo groups[17] which confirms the findings of Scammell and Fregly[22] who have reported that theophylline administration for two weeks increased food intakes in rats. Indeed, Scammell and Fregly[22] observed that theophylline administration to rats caused an increase in both food consumption and faecal bulk. They suggested that theophylline administration may have a direct effect on the thyroid gland or an increase in the sensitivity of the gland to thyroid-stimulating hormone (TSH).[22]

A marked elevation was noted in the activity of CPT in skeletal muscle whereas there was no change in this activity in the liver after theophylline treatment (Tables I and II). Previously we reported that theophylline treatment led to a significantly increase in the levels of free, short-chain acyl, long-chain acyl and total acyl carnitine only in skeletal muscle, heart and kidneys whereas no changes were noticed in the liver as compared to either placebo and control groups.[16,17,21] The reason(s) by which theophylline feeding caused changes in the activity of CPT in the skeletal muscle is unknown. However, there are many possible explanations for the elevation of CPT activity in skeletal muscle. It is known that an important aspect in the regulation of fatty acid oxidation by CPT is the change in the sensitivity of the enzyme to inhibition by malonyl-CoA.[23] It has been reported that adaptive changes occur in both the activity and the response to malonyl-CoA of CPT under different physiological and pathological conditions, such as starvation, diabetes, and hypothyroidism and hyperthyroidism.[24–26] The increase in the activity of CPT in skeletal muscle may demonstrate an increased flow of fatty acid into skeletal muscle for subsequent transferring from palmitoyl-CoA groups to form the corresponding palmitoyl-carnitine groups as catalyzed by CPT. Alternatively, it could be due to theophylline treatment causing significant changes in carnitine distributions in plasma as well as in tissues.[15–17,21]

An increase in the activity of CPT has been suggested to protect cellular metabolism under conditions of abnormal acyl-CoA build up.[27,28] The efficacy of theophylline as a metabolic-inducing agent has been known for several years. For example, the effects of theophylline feeding on fat cells, phosphodiesterase inhibition, release

of intracellularly bound calcium, antagonism to the effects of endogenous adenosine, result in increasing cAMP levels and lipolysis by lipolytic hormones.[14] Therefore, the increase in the concentration of carnitine would help circumvent the enhanced fatty acids that are liberated due to increased lipolytic effects of the theophylline. This is consistent with the observation that significant changes in plasma carnitine concentrations,[16] as well as in cardiac tissues,[17] occurred in the theophylline-treated rats. An increase in the activity of CPT upon theophylline treatment could be due to mobilization of fatty acids with subsequent increase in long-chain fatty acylcarnitine esters in the tissues. The rise in the activity of CPT in the skeletal muscle further supports our contention that theophylline may increase transport of carnitine into tissues and/or may induce carnitine-dependent enzymes.

Results showed that the CPT activity in the liver was 10-fold greater than that in the skeletal muscle which may indicate that theophylline has different action in these two different tissues. The reason(s) for the lack of effect of theophylline treatment on the activity of CPT in the liver is unknown. However, it could be explained by the fact that the hepatic microsomal drug metabolizing system is the site of biotransformation of a variety of substances. Theophylline is rapidly metabolized in the liver by *N*-desmethylation and 8-hydroxylation to methylurates.[29] Only 10% of an administered theophylline dose is reported to be excreted unchanged in urine.[29] Thus an effective concentration of theophylline may not be maintained in the liver to bring about the observed effects on the activity of CPT and its sensitivity to inhibition by malonyl-CoA. Moreover, it has been shown that the activity of CPT in liver is less sensitive to inhibition by malonyl-CoA as compared to either skeletal muscle or cardiac tissues.[25] Thus, liver malonyl-CoA levels are insufficiently low to effect the inhibition of liver CPT, implying that the predominant route of incoming fatty acid in liver is oxidation rather than esterification.[26]

In conclusion, the data showed daily administrated theophylline to adult male rats caused a striking increase in the activity of CPT in skeletal muscle as compared to either control or placebo groups whereas there was no changes in the activity of CPT in hepatic tissues. The observed discrepancies in activity of CPT might be due to there being two isoenzymes, the muscle type M-CPT and liver L-CPT. Therefore, it is possible that theophylline affects only M-CPT activity. It should be noted that we considered the possibility that theophylline treatment interfered with the analytical determination of the activity of CPT. However, estimation of the activity of CPT in tissue homogenate showed that there was no difference in enzyme activity in the presence or the absence of theophylline.

The results raise several points that need further investigations at an intracellular level in tissues and/or key enzyme connected to carnitine. For example, would supplementation of carnitine help in restoring normal enzyme activity to theophylline-treated rats? Does theophylline alter carnitine metabolism, carnitine-dependent enzymes or its distribution in plasma and/or tissues? These questions remain to be tested.

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