L-Carnitine Ameliorates Cancer Cachexia in Mice Partly via the Carnitine Palmitoyltransferase-Associated PPAR-γ Signaling Pathway

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Introduction
Cancer cachexia is a multifactorial syndrome characterized by progressive loss of body weight with the depletion of skeletal muscle and adipose tissue. In cancer cachexia, increased lipolysis and decreased lipogenesis augment the circulating amounts of glycerol and nonesterified fatty acids (NEFAs) [1]. The utilization of NEFAs in the liver is attenuated [2], mainly due to decreased expression levels and activities of carnitine palmitoyltransferases I and II (CPT I and CPT II) which accelerate fatty acid β-oxidation [3, 4]. Additionally, some pro-inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and interferon-γ (IFN-γ) inhibit the lipoprotein lipase, thus preventing adipocytes from storing fatty acids [5].
Carnitine is essential to fatty acid metabolism and is markedly decreased in the serum of cancer cachexia patients [6]. Oral supplementation of L-carnitine has been shown to exert a beneficial effect in cancer cachexia [7] and to reduce the plasma concentration of lipids, including triglycerides [8]. Our previous study has demonstrated that L-carnitine improves cancer cachexia in mice, with a concomitant increase in CPT I and II activities and decreases in serum pro-inflammatory cytokines [3]. However, the mechanisms of the L-carnitine-induced amelioration of cancer cachexia need to be fully elucidated, including the association between the CPT activities and the pro-inflammatory signaling pathways.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate lipid and glucose metabolism. They include three types, known as PPAR-α, PPAR-δ (or -β), and PPAR-γ. PPAR-γ is the key mediator of lipid metabolism [9]. Activation of PPAR-γ stimulates the expression of several genes critical for adipogenesis [9, 10] and also exerts an anti-inflammatory effect by suppressing the nuclear factor-κB (NF-κB) signaling pathway [11]. Several studies have shown that PPAR-γ was decreased dramatically at the mRNA and protein levels in visceral adipose tissues during the development of cancer cachexia [12, 13], suggesting that a hinge of the PPAR-γ signaling pathway in the lipid metabolism is disordered, inducing inflammatory responses in cancer cachexia.

In addition, recent studies have shown that CPT expression levels are decreased in fat and muscle tissues in the development of cancer cachexia, accompanied by the diminishment of PPAR-γ mRNA and protein [14, 15]. Our previous studies have demonstrated that exogenous L-carnitine was able to increase the CPT I and II expression levels and activities in cancer cachexia [3]. Previous studies have also shown that irreversible inhibition of CPT I activities by tetradecyglycidic acid could upregulate the expression of PPAR-γ coactivator 1-α (PGC-1α) in mouse liver [16]. PGC-1α is a nuclear transcription factor and is well known to interact with PPAR-γ, permitting the interaction of PPAR-γ with multiple transcription factors. Thereby, PPAR-γ plays essential roles in fatty acid metabolism and in the anti-inflammatory response [17]. However, the relationship between L-carnitine and PPAR-γ in cancer cachexia, and especially the role of the PPAR-γ signaling pathway, in the ameliorative effects of L-carnitine on cancer cachexia is incompletely known to date. The aim of the present study was to investigate the correlation between CPT I and PPAR-γ and to define the role of the PPAR-γ signaling pathway in the ameliorative effects of L-carnitine on cancer cachexia in a colon-26 tumor-bearing mouse model.

Materials and Methods

Animals and Cachexia Model

The animal experiments were approved by the Institute of Animal Use and Care Committee of Tongji University (Shanghai, China). Cancer cachexia was induced in colon-26 tumor-bearing mice as depicted in our previous study [3].

Groups and Study Protocol

Based on the results obtained in our previous study [3], cancer cachexia was fully developed at day 11 after tumor inoculation and the subsequent interventions were started on day 12. There were in total 42 tumor-bearing mice receiving the treatments designed in this study. 18 tumor-bearing mice were equally randomized into (i) the saline group receiving oral (p.o.) administration of 2 ml saline daily, (ii) the L-carnitine group receiving p.o. administration of 9 mg/kg L-carnitine (catalog no. C0158) daily, and (iii) the L-carnitine + etomoxir group receiving p.o. administration of 9 mg/kg L-carnitine daily and intraperitoneal (i.p.) administration of 20 mg/kg etomoxir (an inhibitor of CPT I; catalog no. E1905) daily.

At the same time, the other 24 tumor-bearing mice were equally randomized into (iv) the pioglitazone group, (v) the GW9662 group, (vi) the L-carnitine + pioglitazone group, and (vii) the L-carnitine + GW9662 group, receiving p.o. pioglitazone hydrochloride (a specific agonist of PPAR-γ; catalog no. E6910) at 10 mg/kg daily, i.p. GW9662 (a selective inhibitor of PPAR-γ; catalog no. M6191) at 1 mg/kg daily, L-carnitine (9 mg/kg p.o.) + pioglitazone (10 mg/kg/day p.o.), and L-carnitine (9 mg/kg p.o.) + GW6962 (1 mg/kg/day i.p.), respectively. 6 healthy mice receiving no treatment were used as normal controls (normal group). L-Carnitine, etomoxir, pioglitazone hydrochloride, and GW9662 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Body weight, food intake, and tumor size (length and width) were measured daily.

At day 19, all mice in experiments 1 and 2 were anesthetized with 2% pentobarbital (40 mg/kg i.p.) and weighed. Physiological cachexia parameters (food intake, body weight, carcass weight, gastrocnemius muscle, and epididymis fat), biochemical parameters (blood glucose, cholesterol, and serum albumin), and serum cytokines (IL-6, TNF-α) were measured. The intact liver was isolated and stored in liquid nitrogen. The remaining intact gastrocnemius muscle and epididymis fat were dissected and weighed.

Measurement of Biochemical Parameters and Cytokines

Blood glucose, serum total cholesterol, and serum albumin were evaluated using an AU2700 automatic biochemical analyzer (Olympus, Japan). Serum TNF-α and IL-6 were detected using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from a portion of the liver and real-time polymerase chain reaction (RT-PCR) analyses were performed as depicted previously [3]. The primers used were as follows: forward 5'-CTG TGG TGG CAT GTG TTC-3' and reverse 5'-AGG GCT CAT CCT GTC TTT-3' for sterol regulatory element-binding protein-1c (SREBP-1c); forward 5'-AGG TCT GTG GTT GCA TGG-3' and reverse 5'-TGC CTG TCA CAG ACG AGC-3' for PPAR-γ; forward 5'-AGA AGC TCA AGC AGA ACC TGA-3' and reverse 5'-ACT TCG TTC AGT CCC TCA ATT-3' for nuclear factor-κB (NF-κB); and forward 5'-TGG ACC TCA GGC CCC TCT-3' and reverse 5'-GCA ACT GAG GGC CTC TCT-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis

All antibodies against the following proteins were used for Western blotting studies: PPAR-α (Abcam, Cambridge, UK; catalog no. ab9349), PPAR-γ (Abcam; catalog no. ab19481), phospho-PPAR-γ (p-PPAR-γ; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog no. sc-28001-R); phospho-p65 (p-p65; Santa Cruz Biotechnology; catalog no. sc-52401); p65 (Abcam; catalog no. ab16502) and β-actin (Abcam; catalog no. ab8227). Total liver protein extracts were obtained and Western blot assays were performed as described previously [3].

Statistical Analysis

All data were expressed as mean ± SD and analyzed using analysis of variance (ANOVA) followed by the least significant difference (LSD) t-test for post-hoc comparison. A P value ≤ 0.05 was considered statistically significant.
Results

Etomoxir Reverses the Effect of L-Carnitine with Respect to Cachexia Parameters, Biochemical Parameters, and Serum Cytokines in Cancer Cachetic Mice

Compared to the animals receiving saline, oral supplementation with L-carnitine at 9 mg/kg/day induced a notable increase in the cachexia parameters (body weight, food intake, carcass weight, gastrocnemius muscle, and epididymis fat) without any obvious effect on the tumor weight. In addition, L-carnitine induced a significant elevation in blood glucose and serum albumin levels and a marked decrease in total cholesterol as well as serum cytokine levels (IL-6 and TNF-α) in cachectic mice. This effect of L-carnitine on cancer cachetic mice was markedly reversed by simultaneous i.p. administration of etomoxir (table 1, www.karger.com/?DOI=439550).

Etomoxir Decreases PPAR-α and PPAR-γ at the mRNA and Protein Levels in Cancer Cachetic Mice Treated with L-Carnitine

L-Carnitine markedly restored the decreased PPAR-α and PPAR-γ expression levels in the cachectic mice receiving saline, both at the mRNA and protein levels. This reversal effect of L-carnitine on the elevated PPAR-α and PPAR-γ expression levels in the mice receiving saline was almost abolished upon etomoxir treatment (fig. 1, www.karger.com/?DOI=439550).

Effects of Pioglitazone or GW9662 on the Cachexia Parameters, Biochemical Parameters, and Serum Cytokines in Cachetic Mice

Compared to the mice receiving saline, L-carnitine or pioglitazone alone induced a notable increase in the cachexia parameters (body weight, food intake, gastrocnemius muscle, and epididymis fat) and serum albumin, and a notable decrease in the total cholesterol as well as serum cytokine levels (IL-6, TNF-α). Except for the effect on body weight, these effects of L-carnitine were markedly attenuated by GW9662, a selective inhibitor of PPAR-γ (table 2, www.karger.com/?DOI=439550).

Pioglitazone or GW9662 Changes the mRNA Expression Levels of SREBP-1c and FAS in the Livers of Cachetic Mice

The SREBP-1c and FAS mRNAs were expressed at baseline levels in the normal mice; the levels of these mRNAs were increased in the livers of cachetic mice, and were markedly decreased by pioglitazone or L-carnitine. This effect of L-carnitine on the expression levels of SREBP-1c and FAS was significantly reversed by GW9662 (fig. 2, www.karger.com/?DOI=439550).

Pioglitazone or GW9662 Changes the Protein Expression Levels of p-PPAR-γ, p-p65, and COX-2 in the Livers of Cachetic Mice

Compared to the normal controls, the protein expression levels of p-p65 and cyclooxygenase (COX)-2 were notably increased, while the degree of phosphorylation of PPAR-γ was markedly decreased in the cachectic mice receiving saline. This expression profile of these proteins was nearly reversed by L-carnitine or pioglitazone alone. However, these reversal effects of L-carnitine and pioglitazone were markedly attenuated by GW9662 (fig. 3, www.karger.com/?DOI=439550).

Discussion

Although L-carnitine could ameliorate the mentioned symptoms of cancer cachexia [7], little is known about the mechanisms of action of L-carnitine in improving cancer cachexia. The current results showed that regulation of PPAR signaling by L-carnitine is associated with the CPT I activities, and that the PPAR-γ signaling pathway might be involved in the amelioration effect of L-carnitine on cancer cachexia.

The impairment of β-oxidation of fatty acids in the liver plays an essential role in the development of cancer cachexia [18]. CPT is the key regulator of β-oxidation of fatty acids, and both the CPT expression and activity in the liver were markedly decreased during the development of cancer cachexia, suggesting that CPT may be a potential therapeutic target for cancer cachexia. The present study shows that p.o. administration of L-carnitine improved the cancer cachexia and biochemical parameters in the cachetic mice, which is consistent with the results from our previous study. Etomoxir, an inhibitor of CPT I, attenuated the ameliorative effect of L-carnitine on cancer cachexia, implying that L-carnitine improved the cancer cachexia symptoms in an at least CPT-dependent manner.

Inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFN-γ, could accelerate the development of cancer cachexia [19]. TNF-α-mediated inflammation was associated with CPT activity [20], but it is not clear whether the inflammatory reaction is regulated by CPT. In our study, etomoxir, an inhibitor of CPT I activity, abated the decrease in the serum TNF-α and IL-6 levels induced by L-carnitine, suggesting that L-carnitine could increase the CPT activity to lessen the serum concentrations of TNF-α and IL-6, thus ameliorating cancer cachexia. In contrast, although the decrease induced by L-carnitine in the serum concentrations of IL-6 and TNF-α was statistically significant, the absolute decreased value was low, suggesting a slight effect of L-carnitine on the inflammation reaction in the livers of cancer cachetic mice. Previous studies have demonstrated that the ameliorative effects of L-carnitine in cancer cachexia show a dose-dependent pattern [21]. Therefore, the slight effect of L-carnitine may be explained by the lower dose used in this study.

Accumulating evidence has shown that PPAR-γ activation exerts an anti-inflammatory effect in the liver [22] and reduces the TNF-α and IL-6 expression levels in pancreatic islet cells [23]. In our study, L-carnitine decreased the serum concentrations of TNF-α and IL-6, concomitant with a notable increase in PPAR-γ at the mRNA and protein levels. This effect was markedly lessened by etomoxir administration, suggesting that L-carnitine regulated the CPT activities to modulate the PPAR-γ signaling pathway, which might be involved in the decreased serum concentrations of TNF-α and IL-6 by L-carnitine.

To further explore the role of PPAR-γ in the improving effect of L-carnitine on cancer cachexia, pioglitazone and GW9662 were used in this study to activate and inhibit PPAR-γ, respectively. The transcription factor SREBP-1c and FAS are well established to be regulated by PPAR-γ in fatty acid synthesis [24, 25]. In this study, the expression levels of the genes coding for these two proteins
were markedly elevated in the livers of cachectic mice, and were markedly decreased by pioglitazone or L-carnitine. This effect of L-carnitine on the expression levels of SREBP-1c and FAS was significantly reversed by GW9662. The current findings suggest that L-carnitine could regulate the PPAR-γ signaling pathway involved in inhibiting fatty acid synthesis in the livers of cachectic mice. In addition, GW9662 abated the improvement due to L-carnitine in the cachexia and biochemical parameters in cancer cachectic mice, suggesting that L-carnitine ameliorates cancer cachexia via the PPAR-γ-dependent signaling pathway.

Pro-inflammatory cytokines such as TNF-α and IL-6 play essential roles in cancer cachexia by activating NF-κB signaling [26, 27]. PPAR-γ could directly bind to p65 or p50 [28], or it could inhibit the degradation of the inhibitor of NF-κB (IκB) to inactivate NF-κB signaling [29]. In our study, increased levels of phosphorylated p65 were noticeable in the livers of cachectic mice receiving saline, which was attenuated by pioglitazone alone, suggesting that PPAR-γ is a negative upstream regulator of the NF-κB p65 signaling pathway under cancer cachexia conditions. However, the exact mechanisms need to be explored in detail. In this study, similar to pioglitazone, L-carnitine could activate the PPAR-γ-dependent signaling pathway. Together with the results mentioned above, our findings in this study indicate that L-carnitine could activate CPT-associated PPAR-γ to suppress the NF-κB p65 signaling pathway, thus exerting an anti-inflammatory effect to improve cancer cachexia.

A previous study demonstrated that L-carnitine at 400 mg/kg could markedly inhibit in vivo tumor growth in HepG2 tumor-bearing mice [21], which is inconsistent with the current findings that L-carnitine did not affect the tumor weight. This might be explained by the different animal model and L-carnitine dosage used in our experiment. Interestingly, pioglitazone was recently demonstrated to reduce the final tumor mass and to increase survival in cachectic rats, accompanied by upregulating PPAR-γ gene expression [30]. In the present study, L-carnitine did increase the expression levels of PPAR-γ at both the mRNA and protein levels. Whether L-carnitine elevates the survival rate in cancer cachexia needs to be explored in future experiments.

One limitation of the present study is that we did not explore the effects of L-carnitine on the activities of PPAR-γ, especially in the normal mice. Thus, the exact correlation between L-carnitine and the PPAR-γ/NF-κB signaling pathway remains unclear and needs to be fully elucidated in the future.

References


Online Supplemental Material

Fig. 1. Effects of etomoxir on the mRNA and protein expression levels of SREBP-1c and FAS in the livers of cancer cachectic mice.

Fig. 2. Effects of pioglitazone or GW9662 on the mRNA expression levels of SREBP-1c and FAS in the livers of cachectic mice.

Fig. 3. Effects of pioglitazone or GW9662 on the protein expression levels of phospho-PPAR-γ, phospho-p65, and COX-2 in the livers of cancer cachectic mice.

Table 1. Effects of etomoxir on the cachexia parameters, biochemical parameters, and serum cytokines in cachectic mice treated with L-carnitine

Table 2. Effects of pioglitazone or GW9662 on the cachexia parameters, biochemical parameters, and serum cytokines in cachectic mice treated with L-carnitine

To access the online supplemental material, please refer to www.karger.com/DOI=439550.

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Disclosure Statement

The authors have declared that no competing interests exist and that they have no financial interest in the subject matter or matters discussed in this manuscript.


