博士論文

論文題目

Studies on serum L-carnitine level and its regulatory factors in the cat
（猫の血中 L―カルニチンとその制御因子に関する研究）

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Doctoral Dissertation

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Contents

List of Abbreviations

BCS: body condition scoring

CACT: carnitine-acylcarnitine translocase

CoA: coenzyme A

CPT: carnitine-palmityoltransferase

DM: diabetes mellitus

DMEM: Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

FSkMC: feline skeletal muscle cell

GFR: glomerular filtration rate

GI: gastrointestinal

H_2O_2: hydrogen peroxide

HL: hepatic lipidosis

NEFAs: nonesterified fatty acids

OCTN: organic cation/carnitine transporter

PPAR: peroxisome proliferator-activated receptor

ROS: reactive oxygen species

SAA: serum amyloid A

UPS: ubiquitin proteasome system

Abstract

Background and Aim

L-carnitine is an essential nutrient needed in fatty acid metabolism that enables the translocation of long-chain fatty acids into the mitochondria. L-carnitine is actively transported *via* organic cation/carnitine transporter (OCTN)-2 into cells. In mammals, L-carnitine is usually obtained from dietary sources and biosynthesis in the body, and the skeletal muscle in the major tissue reservoir of L-carnitine. L-carnitine has been extensively used as a nutritional supplement in humans for losing body fat and/or avoiding muscle wasting. As well as in humans, metabolic problems in cats including obesity and wasting have been considered to be important health issues during recent decades. L-carnitine supplementation to obese cats has been tried and was proved to accelerate body fat loss. However, there is still limited information about L-carnitine metabolism in cats. Recently, researchers have been focusing on the blood levels of L-carnitine to assess the relationship between L-carnitine metabolism and certain pathophysiological conditions. Serum L-carnitine concentrations elevate in human patients with hepatic cirrhosis, malignancy and chronic heart failure, and also in dogs with hepatic disorders. On the other hand, the significance of serum L-carnitine concentrations in cats is entirely unknown. The present study was designed to obtain basal information about L-carnitine levels in the feline serum, and to elucidate the underlying mechanisms those change the L-carnitine levels. Chapter I describes serum concentrations of total, free and acylated L-carnitine in the sera of clinically healthy cats and diseases cats. Chapter II deals with *in vitro* models of the skeletal muscle to simulate the release of L-carnitine from skeletal muscle cells under oxidative or

hypoxic conditions. In chapter III, a microarray technique was employed to analyze the gene expressions of murine skeletal muscle cells under oxidative or hypoxic stress *in vitro* to reveal the mechanism of L-carnitine release from cells.

Chapter I: Serum L-carnitine levels in healthy and diseased cats

Firstly, serum total, free and acylated L-carnitine levels were measured in 41 clinically healthy cats at various ages by using commercial kits on an automatic biochemical analyzer. A positive correlation was found between the cats' ages and the serum acylcarnitine levels, whereas serum total or free L-carnitine concentrations showed little age-related changes. Secondly, serum total, free and acylated L-carnitine levels were measured in 139 randomly selected feline patients with various diseases presented at Veterinary Medical Center, the University of Tokyo between 2006 and 2012. Total and acylated L-carnitine concentrations were significantly higher in cats with diabetes mellitus, neoplastic disorders and cardiac disorders than those in healthy cats. Thirdly, relationships between serum L-carnitine levels and disease markers (serum amyloid A and haptoglobin as acute phase proteins; leptin as a marker of cachexia) were analyzed in the diseased cats. A weak but significant correlation between serum total L-carnitine and haptoglobin was confirmed. Thus, the increased serum L-carnitine, which might come from the skeletal muscle, was considered to reflect insulin resistance, inflammatory events and/or oxidative stress in cats with diabetes mellitus, malignancy and heart failure, respectively. Also, the data suggest the possibility of serum L-carnitine levels as a positive disease biomarker in cats.

Chapter II: *In vitro* genetic analysis of L-carnitine release mechanism from murine skeletal myotubes under oxidative or hypoxic stress

To simulate the L-carnitine release from the skeletal muscle under oxidative and/or hypoxic conditions, *in vitro* models were employed. Mouse C_2C_{12} myoblasts and commercially available primary cultures of feline skeletal myocytes were used for this aim. These cells were differentiated into myotubes and pre-cultured in culture media supplemented with 250 mM L-carnitine to construct the intracellular L-carnitine pools. Cellular L-carnitine uptake was blocked by OCTN2 inhibitors (amiodarone, carvedilol, propantheline and verapamil), indicating the myotubes well presented OCTN2. After that, mouse and feline myotubes were cultured under an oxidative model (cells cultured in the presence of 0.5 mM H_2O_2 for 12 hours) or a hypoxic model (cells cultured under 100% nitrogen atmosphere for 12 hours), and the intra- and extra-cellular L-carnitine amount was measured. Both in the oxidative and hypoxic models, release of L-carnitine into the extracellular space was accelerated. Additionally the OCTN2 inhibitors had no inhibitory effect on the L-carnitine release from the myotubes. In this chapter, the disease-related release of L-carnitine from the skeletal muscle was reproduced at least in part *in vitro*, however, the efflux pathway of L-carnitine could not be identified. Intramyocellular L-carnitine might leak *via* passive permeability and/or unknown route.

Chapter III: *In vitro* genetic analysis of L-carnitine release mechanism from murine skeletal myotubes under oxidative or hypoxic stress

To reveal the mechanism of L-carnitine release from murine C_2C_{12} myotubes, a microarray technique was employed to analyze the gene expressions under the same oxidative or hypoxic conditions as in Chapter 2. Significant up-regulation of *Mrpl52* and down-regulation of *RNF220* were the major changes, indicating strong expression

of mitochondrial transcript and suppression of the ubiquitin proteasome system, respectively. On the other hand, no significant changes in gene expression related to L-carnitine metabolism was observed. Despite the definite mechanism of intramyocellular L-carnitine release remained unclear, certain changes in mitochondrial function during energy loss might affect the maintenance of the L-carnitine pools.

Conclusion

This study revealed the increase in feline serum L-carnitine level in diseased cats. The increased level was considered to reflect the L-carnitine release from the skeletal muscle accompanied by insulin resistance, inflammatory events and/or oxidative stress. The release of intramuscular L-carnitine under oxidative and/or hypoxic conditions may be *via* unknown pathways including passive permeability, and may be caused by muscular energy loss.

To date, L-carnitine has been supplemented to healthy and diseased cats with little evidence and little consideration. From the standpoint of this study, in diseased cats especially with diabetes mellitus, malignancy and heart failure, the serum L-carnitine level seems to be saturated by the release of L-carnitine from the skeletal muscle. To utilize L-carnitine for diseased cats, appropriate consideration about the L-carnitine metabolism would be needed.

General introduction

General information

L-carnitine (L-β-hydroxyl-γ-*N*-trimethylaminobutyric acid), derived from "carnus" in Latin meaning fresh, was first discovered in muscular tissues over a century ago, and its chemical structure $C_7H_{15}NO_3$ was established in 1927 by Tomita and Sendju [123]. Despite there are two existing enantiomers of L-carnitine (L- and D-carnitine), only the L-isomer is biophysiologically active [88, 123].

L-carnitine, presenting as free L-carnitine and as esterified derivatives acylcarnitine, is primarily obtained from dietary sources, and also via endogenous biosynthesis [116]. In mammals, over 90% of L-carnitine in the body is present in the skeletal muscle, and the rest of L-carnitine is distributed in the heart, liver, extracellular fluid and kidney [88]. Orally ingested L-carnitine is taken up in the small intestine mainly via the active sodium-dependent organic cation/carnitine transporter 2 (OCTN2). Unabsorbed L-carnitine is degraded in the large intestine by indigenous flora into γ-butyrobetaine and trimethylamine. Trimethylamine is absorbed and further converted to trimethylamine-N-oxide, which contributes to cardiovascular disease risks, indicating the possible side effect of red meat consumption including large amount of L-carnitine [57, 88]. L-carnitine is synthesized ultimately from the amino acids lysine and methionine mainly in the liver [117]. This synthesis process depends on the presence of some vitamins including vitamin B6, ascorbic acids, nicotinic acid and folate as coenzymes in the biosynthetic pathway [117, 123]. The primary route of systemic L-carnitine elimination is via renal excretion and its filtration clearance is almost equal to the glomerular filtration. Therefore most filtered L-carnitine is

reabsorbed by the proximal tubule and returned into the bloodstream in order to reduce L-carnitine loss and maintain endogenous concentration [88].

Biological functions of L-carnitine

The amphiphilic property of L-carnitine enables the translocation of long-chain fatty acids through the mitochondrial membranes followed by the β oxidation [123]. Figure 1 shows the major function of L-carnitine: to bring the acyl groups carried by fatty acids into mitochondria to generate energy. Other functions of L-carnitine contain modulation of acyl-CoA/CoA ratio, storage of acetylcarnitine as energy source, and remove of toxic effects of poorly metabolized acyl groups via renal excretion [117].

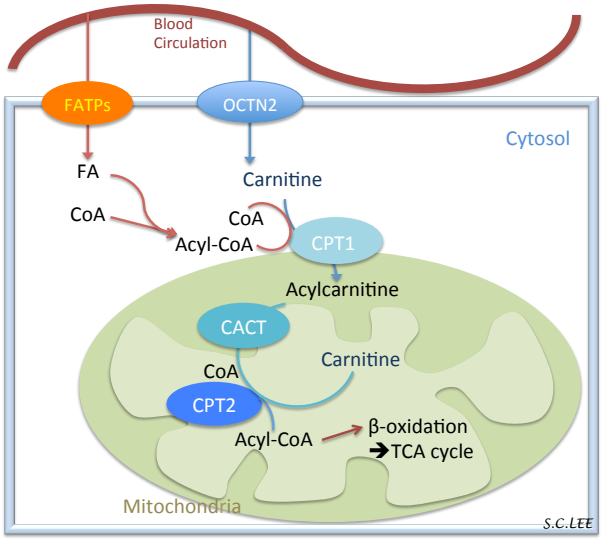


Figure. 1. The major function of L-carnitine in transportation of long-chain fatty acids. Fatty acids and L-carnitine in the circulation are taken up into the cell through fatty acid transport proteins (FATPs) and OCTN2. In the cytosol, acyl groups carried by fatty acids are transferred to coenzyme A (CoA) at first, and then brought into mitochondria by being trans-esterified to L-carnitine as acylcarnitine through carnitine palmitoyltransferase 1 (CPT1) at the outer mitochondrial membrane. Acylcarnitine can cross the inner mitochondrial membrane via its carrier: carnitine-acylcarnitine translocase (CACT). At the matrix inside mitochondria, the acyl groups are transferred back to intramitochondrial CoA through the reaction catalyzed by carnitine palmitoyltransferase 2 (CPT2). Thus acyl groups carried by long-chain fatty acids can

finally enter β oxidation in the form Acyl-CoA, and the derivatives can further enter tricarboxylic acid (TCA) cycle to generate energy.

Protection against oxidative stress

Antioxidative activities of L-carnitine have been drawing scientists' attention. L-carnitine is reported to scavenge free reactive oxygen species (ROS) and to chelate heavy metals for promoting endogenous antioxidant defense and inhibiting lipid peroxidation [40]. The protection of L-carnitine against oxidative stress is involved with up-regulation of peroxisome proliferator-activated receptor alpha (PPARα), a ligand-activated transcription factor regulating lipid metabolism and energy hemostasis [40, 61]. PPARα is abundantly expressed in muscles, heart and liver [5], while these organs are also the primary sites of L-carnitine storage. Studies show that fasting, energy restriction and PPARα agonist treatment in rats lead to elevated hepatic L-carnitine concentration due to activation of PPARα, which stimulates L-carnitine biosynthesis and increase of OCTN2 mRNA concentration through up-regulation of the mitochondrial and peroxisomal genes involved in fatty acid catabolism such as CPT1and CPT2 [27, 115]. Considering mitochondria is where L-carnitine functions and also the major place where endogenous ROS are produced, impaired mitochondrial function is closely connected to L-carnitine-related pathophysiology. Insulin resistance is the most important consequence of mitochondrial dysfunction, and its related disorders mainly comprise type 2 DM and heart failure [69].

Role of L-carnitine in diseases

Primary L-carnitine deficiency caused by impairment of L-carnitine transporter OCTN2 occurs in 0.1~0.5‰ of human populations while secondary L-carnitine

deficiency caused by genetic defects or acquired problems like renal or hepatic disorders is less severe but much more common [30]. The deficiency of L-carnitine mainly manifests as skeletal muscle myopathy, cardiomyopathy and encephalopathy, but is also strongly associated with hepatic and endocrinal disorders [30, 103].

Dietary L-carnitine supplementation is widely used for increasing physical performance, anti-obesity, anti-wasting and anti-aging, also as complimentary therapy for uremia, diabetes mellitus (DM), hepatic disorders, neuromuscular and cardiovascular diseases in humans, horses and small animals, but the benefits can be varied [8, 20, 30, 33, 42, 91, 124]. Supplementation of L-carnitine-included nutrients can ameliorate insulin-mediated glucose disposal and improve skeletal mitochondrial dysfunction, also can prevent muscle wasting under pathological conditions [91, 101].

Serum L-carnitine profile

Serum L-carnitine concentration is higher in men than women as same sexual dimorphism is noted in rats, and it's still controversial whether serum L-carnitine is under age-related impact or not in humans [9, 80, 89]. Although L-carnitine deficiency is associated with dilated cardiomyopathy and lipid storage myopathy in dogs [34, 92, 100], no definite feline case has been reported yet.

Hypercarnitinemia had been reported in hepatic cirrhosis and blood L-carnitine concentration was found elevated during fasting in human, leading to the discussions about relationship between plasma L-carnitine concentration and liver damage degree [1, 21, 46]. Significant L-carnitine elevation is also reported in canine hepatopathy, suggesting L-carnitine concentration may be an indicator of hepatic disorders or related symptoms [77].

L-carnitine in cats

As lifespan of companion animals lengthens, obesity continuously affects the middle-aged cats but now weight loss becomes a typical future of the geriatric cats [104]. Excessive carbohydrates in commercial cat foods are supposed to contribute to the increasing incidence of obesity, insulin resistance and diabetes mellitus (DM) in domestic cats [118]. In their natural habit, cats consume prey high in protein and rely on nutrients in animal tissues to meet their specific nutritional requirements. High protein diet is considered to more closely mimic the cat's natural diet and should benefit cats for their strict carnivore nature. However, studies showed vague effect of the high protein diet on weight loss in healthy cats [118].

Obese cats given high protein diet have been reported to lose greater proportion of body fat and smaller proportion of lean body mass, and the probable mechanism is suppression of non-esterified fatty acids (NEFAs) in the blood [44, 116]. Therefore these facts bring an idea that the beneficial effect of high protein diet might not rely on protein itself but come from L-carnitine, a well-known necessary substance also rich in food from animal source, which plays a crucial role in fatty acid metabolism. Besides weight loss, supplementation of L-carnitine in obese cats is proved to have similar effect as high protein diet that it facilitates weight loss in overweight cats with increase in the lean body mass and decrease in the fat mass [11, 12]. Therefore it might have achieved the expected effects that high protein diets in cats failed to reach.

Jacobs demonstrated L-carnitine concentrations in kitten and young adult cats in 1990, but L-carnitine data of old cats haven't been published [50]. Positive correlation between plasma and hepatic L-carnitine concentrations was reported in cats, while

plasma L-carnitine concentration correlated with nutritional intake of L-carnitine in healthy humans [50, 106]. Furthermore, the reported feline serum L-carnitine concentration is lower than humans, while the feline tissue L-carnitine concentration is similar to humans [50, 80, 124].

L-carnitine in feline diseases

Although L-carnitine is clinically used as an interventional nutrition in myocardiopathy and hepatic lipidosis (HL) for relating to L-carnitine deficiency in human patients, L-carnitine concentrations of idiopathic HL cats are higher than healthy individuals with unclarified mechanism [30, 49, 88]. Significant hepatic lipid accumulation happens during whether weight gain or weight loss in cats [48]. As one of the consequences of anorexia especially in obese cats, HL is a frequently diagnosed hepatobiliary disease with potential contributing factors including pre-existing insulin resistance and obesity [44, 71]. In cats with HL, both higher serum NEFAs and increased serum L-carnitine concentration were reported, suggesting the possibility of endogenously responded L-carnitine elevation through *de novo* synthesis for accelerating fatty acid metabolism [8, 49]. Besides, previous researches failed to support the hypothesis that L-carnitine deficiency leads to fatty acids accumulation in the liver [3], also failed to establish the relationship between L-carnitine deficiency and idiopathic HL together [49].

So far, L-carnitine is involved with a variety of human diseases [30]. However, the role of L-carnitine in feline pathophysiology has not been well studied, yet there is still very limited information about L-carnitine and feline diseases.

Chapter I

Serum L-carnitine levels in healthy and diseased cats

Summary

L-carnitine is well known as an essential component that enables the translocation of long-chain fatty acids into the mitochondria. However, there has been poor information about L-carnitine metabolism in feline pathophysiology. The aim of this study is to evaluate the circulating L-carnitine levels in healthy and diseased cats. Total, free and acylated L-carnitine concentrations in the sera from 41 clinically healthy cats and 139 diseased cats were measured, and the correlation between L-carnitine levels and serum amyloid A (SAA), haptoglobin and leptin was estimated. In the healthy cats, significantly positive correlation was found between age and acylcarnitine level, while there was no age-related changes in total or free L-carnitine concentrations. Serum L-carnitine concentrations elevated in cats with DM, neoplastic and cardiac disorders. Serum total, free and acylated L-carnitine concentrations weakly but significantly correlated with serum haptoglobin level, but not with SAA or serum leptin concentrations. Hence the disease-related hypercarnitinemia might reflect the systemic acute phase reaction. This study, at least in part, may suggest the potential of serum L-carnitine level as a positive disease marker in cats.

1.1. Introduction

L-carnitine is well known as an essential substance that enables the translocation of long-chain fatty acids into the mitochondria, therefore plays a crucial role in fatty acid metabolism [124]. Dietary supplementation with L-carnitine is proved to promote weight loss, facilitate fatty acid oxidation, improve liver function and reduce plasma NH_3 in obese cats [8, 11, 12]. However, there has been poor information about L-carnitine metabolism in aged or diseased conditions in cats.

Human plasma L-carnitine concentration is generally considered to be 40-50 μM [88]. However, serum L-carnitine concentration in cats is lower than in humans while their tissue L-carnitine concentrations are similar [50, 80]. L-carnitine concentrations in adult cats are greater than kitten, and elevation of serum L-carnitine concentrations in cats with idiopathic HL is reported with increased *de novo* synthesis [8, 50]. Hypercarnitinemia is also reported in human patients with HL and dogs with hepatic disorders [1, 21, 46, 77], indicating the possibility of responsive L-carnitine elevation. L-carnitine can be endogenously biosynthesized by the liver, which also modulates acute phase proteins synthesis [26, 81].

The initial purpose of this chapter is to understand the age-associated change and disease-dependent differences of feline serum L-carnitine, and to help understanding the role of L-carnitine in the cat. Therefore, serum total L-carnitine, free L-carnitine and acylcarnitine concentrations were measured to compare with markers of diseases to make it clear if L-carnitine concentration is a disease-related pattern. SAA and haptoglobin as major feline acute phase proteins were chosen to indicate inflammation [13, 51]. SAA indicates inflammation and its elevation is noted in several

diseases, also SAA is considered a prognostic marker in diseased cats [94, 114]. Haptoglobin is a moderate acute phase protein for lesser elevation on stimulation in cats, and is considered to associate with interactions of health status [26, 51]. Furthermore, haptoglobin is also a marker for adiposity in humans [15]. Serum leptin was selected to represent further nutritional status or inflammation-associated cachexia [14, 67]. Additionally, serum leptin concentration is reported to positively correlate to BCS in clinically healthy cats [71].

1.2. Materials and methods

Animals

Sera from 41 clinically healthy cats including 30 domestic shorthair cats and 11 Siamese cats, 21 males and 20 females, between 1 month- to 10 year-old were kindly gifted from Oriental Yeast Co., Ltd. (Tokyo, Japan). Sera from 139 client-owned feline patients were collected in Veterinary Medical Center, the University of Tokyo from April 2006 to March 2012. Based on the medical records, diseased cats were divided into 9 subgroups including DM (n=18), cholangitis/ hepatitis/pancreatitis (n=12), renal/urinary tract disorders (n=8), neoplastic disorders (n=39), gastrointestinal (GI) disorders (n=11), viral disorders (n=10), cardiac disorders (n=8), hematological disorders (n=16) and others (n=17). The cholangitis/hepatitis/pancreatitis subgroup included cholangitis (n=3), pancreatitis (n=3), hepatitis (n=2), biliary obstruction (n=1), HL (n=1), hepatic cyst (n=1) and portal systemic shunt (n=1). The renal/urinary tract disorder subgroup included cystitis (n=2), renal lithiasis (n=1), renal dysplasia (n=1) and polycystic kidney disease (n=1). The neoplastic disorder subgroup consisted of malignant lymphoma (n=20), adenocarcinoma (n=7), squamous cell carcinoma (n=4), undiagnosed abdominal tumor (n=3), mast cell tumor (n=2), gastrointestinal stromal tumor (n=1), pulmonary (n=1) and brain tumor (n=1). GI disorders consisted of gastritis (n=3), stomatitis (n=1), enteritis (n=1), colitis (n=1), foreign body (n=1), eosinophilic granuloma (n=1), giardiasis (n=1), coronavirus infection (n=1), and intestinal perforation associated peritonitis (n=1). Viral disorders comprised feline infectious peritonitis (n=7), feline immunodeficiency virus (FIV) infection (n=1) and FIV/feline leukemia virus coinfection (n=2). Cardiac disorders comprised hypertrophic

cardiomyopathy (n=6) and chronic heart failure (n=2). Hematological disorders include immune-mediated hemolytic anemia (n=5), pure red cell aplasia (n=2), anemia (n=2), myelodisplastic syndrome (n=2), acute lymphoblastic leukemia (n=1), multiple myeloma (n=1), fragiocytosis (n=1), thrombosis (n=1) and haemobartonellosis (n=1). Others included skin diseases (n=4), neurologic disorders (n=3), pneumonia and rhinitis (n=3), diaphragmatic and hiatal hernia (n=2), malnutrition (n=1), hyperthyroidism (n=1), pyruvate kinase deficiency (n=1), ovarian cyst (n=1), and asthma (n=1).

Analysis of serum L-carnitine, SAA, haptoglobin and leptin concentrations

Measurement of serum L-carnitine, SAA, haptoglobin and leptin were performed with Total Carnitine Assay Kit and Free Carnitine Assay Kit (Kainos Laboratories, Inc., Tokyo, Japan) using a Model 7180 automated clinical chemistry analyzer (Hitachi High-Technologies, Tokyo, Japan). Acylcarnitine concentration was assumed to be equal to total L-carnitine concentration minus free L-carnitine concentration. SAA was measured with a commercial reagent for human SAA (LZ-SAA, Eiken Chemical Co. Ltd., Tokyo, Japan) and haptoglobin was measured by Haptoglobin (Feline) ELISA kit (American Laboratory Products Co. Ltd., New Hampshire, USA) [113]. Leptin was measured using Cat Leptin (LEP) ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, China). Total L-carnitine, free L-carnitine and acylcarnitine concentrations were compared between different ages in control group and between different diseases in disease group. Also L-carnitine concentrations were also compared with estimate of SAA, haptoglobin and leptin to figure out their connections.

Statistic analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to analyze age-dependent difference in the control group. Comparisons between different genders, healthy cats under or over 7 years old, also between the control group and all disease subgroups were analyzed by unpaired t-test or Mann-whitney test depending on the result of Kolmogorov-Smirnov test. Correlation of L-carnitine concentrations with age in healthy cats and L-carnitine concentrations with haptoglobin, SAA or leptin in all cats were analyzed by Pearson's correlation coefficient. GraphPad Prism for Macintosh OS ver. 5 was used and obtained p value under 0.05 was considered to be statistically significant.

1.3. Results

Serum L-carnitine concentrations in healthy cats at different ages were shown in Figure 1. There was no obvious difference of total and free L-carnitine between different ages or genders, but there was a significantly positive correlation between acylcarnitine concentration and age. Also acylcarnitine level in cats over 7 years of age was significantly higher than the younger cats (p = 0.024). In male cats, total L-carnitine level in geriatric cats over 9 years of age was significantly higher than kittens under 3 months of age. As in diseased cats, significant elevation of total L-carnitine in cats with DM, neoplastic and cardiac disorders, significant elevation of free L-carnitine in cats with DM, cholangitis/hepatitis/pancreatitis, renal/urinary tract disorders, neoplastic, viral and cardiac disorders, and significant elevation of acylcarnitine in cats with DM, neoplastic disorders, GI and cardiac disorders were found (Table. 1). The SAA, haptoglobin and leptin data in cats were also showed in Table 1. Correlation between total L-carnitine and SAA, haptoglobin and leptin concentrations were showed in Figure 2. L-carnitine levels including total, free L-carnitine and acylcarnitine were significantly correlated to haptoglobin.

1.4. Discussion

In the present study, we assessed the age- and sex-related changes in serum L-carnitine concentrations in cats. A positive correlation between age and serum acylcarnitine level was found thus acylcarnitine level in cats over 7 years of age was significantly higher than the younger cats. Besides, an age-related change showed in male cats that total L-carnitine level in geriatric male cats aged over 9 years was higher than male kittens under 3 months of age. It was reported that plasma L-carnitine values were higher in female cats than in male adults [50], but we failed to identify gender-associated differences in serum L-carnitine concentrations. In human medicine, female serum L-carnitine concentration increased with age while muscle L-carnitine level remained constant as aging, while male serum L-carnitine concentration stayed stable but muscle L-carnitine level drops with age [80].

Elevation of blood acylcarnitine was related to insulin resistance in obesity and type 2 DM [7, 72, 78]. Also blood acylcarnitine level was inversely correlated with physical performance in the elderly [66] and with glomerular filtration rate (GFR) therefore associated with renal insufficiency [31, 36]. Besides, insulin resistance might have explained the relationship between acylcarnitine and DM, neoplastic and cardiac disorders [96, 97]. In addition, insulin resistance was a putative early marker of mitochondrial carnitine-dependent dysfunction [87]. It is intriguing that acylcarnitine elevation in cats is significant in GI disorders which theoretically lead to malabsorption of carnitine, while lower serum level of acylcarnitine is reported in inflammatory bowel disease and gastrointestinal cancer cachexia patients [23, 68]. Taken together, the elevation of acylcarnitine level in aged cats could indicate

increased insulin resistance, decreased physical performance and decreased GFR.

Serum total L-carnitine concentration increased in cats with DM, neoplastic and cardiac disorders. L-carnitine elevation was reported in cats with idiopathic hepatic lipidosis and dogs with hepatopathy [50, 77]. Also in human medicine, hypercarnitinemia was found in patients with hepatic cirrhosis, idiopathic hypertrophic cardiomyopathy, heart failure and cancer [1, 21, 75, 110, 119]. L-carnitine elevation in heart failure and cancer patients was assumed to be the consequence of muscular carnitine losing from skeletal muscle for muscular L-carnitine concentration reduction correlated with plasma L-carnitine concentration increase [105, 110, 119]. Yet plasma L-carnitine level correlated with muscle strength and reflects muscle atrophy with age in female haemodialysis patients [19]. As weakness and muscle atrophy caused by hyperglycemic neurological dysfunction was a common complication in diabetic cats [73], L-carnitine elevation in DM cats might also be involved with muscular damage.

On the other hand, considering mitochondrion is where carnitine functions and also where endogenous ROS is produced, impaired mitochondrial function could connect to L-carnitine-related pathophysiology. Besides oxidative stress, insulin resistance is the most important consequences of mitochondrial dysfunction, and its related disorders mainly comprise type 2 DM and heart failure [69], also insulin resistance may contribute to cancer initiation and progression [2]. Supplementation of L-carnitine-included nutrients can ameliorate insulin-mediated glucose disposal and improve skeletal mitochondrial dysfunction, also can prevent muscle wasting under pathologic conditions [91, 101]. Therefore L-carnitine level change in diseased cats with DM, neoplastic and cardiac disorders might associate with insulin resistance, too.

It's an novel finding that elevation of serum L-carnitine concentrations were correlated with haptoglobin, which is one of the major acute phase proteins in several mammal species including the cat, also an endogenous antioxidant for scavenging free hemoglobin induced oxidative activity, but supposed to play the minor role compared to SAA and α-1 acid glycoprotein in cats based on the magnitude of response [13]. In the present study, serum haptoglobin increased significantly in DM cats, which hasn't been reported. Feline DM is similar with human type 2 DM for sharing the crucial feature: pancreatic islet amyloidosis [79]. Also haptoglobin purified from human plasma was proved to suppress deposit formation of amyloid fibrils and to inhibit amyloid formation even in the form of haptoglobin-hemoglobin complex [122]. Therefore the responsive elevation of haptoglobin was predictable in diabetic cats. Additionally, canine haptoglobin concentration dropped after weight loss accompanied by decreases in C-reactive protein and insulin: glucose ratio, also serum haptoglobin indicated adiposity in humans [15, 35]. Since both L-carnitine and haptoglobin were mainly biosynthesized in the liver, the elevation of L-carnitine level and its correlation to haptoglobin suggested the potential of serum L-carnitine concentrations as a disease marker in cats as other liver-generated acute phase reactant. Furthermore, L-carnitine supplementation has been reported to reduce SAA, C-reactive protein and interleukin-6 in hemodialysis human patients [99, 109, 111], suggesting the antioxidant role of L-carnitine under pathological conditions.

Although not correlated with L-carnitine concentrations, significant decreased serum leptin concentration in diseased cats was observed in this study. Lipid-infusion was reported to induce serum leptin elevation in cats [102]. In humans, blood leptin

concentration increased in diseases such as cardiovascular disease, pancreatitis and chronic kidney disease while hypoleptinemic state was generally caused by starvation [52, 70, 76, 82]. Thus the reason for serum leptin reduction in diseased cats could be anorexia. Also evidences showed the importance of circulating leptin level in anorexia-cachexia [28].

1.5. Conclusion

In the present study, a positive correlation between age and the serum acylcarnitine level was found, and also acylcarnitine level in cats over 7 years of age was significantly higher than younger cats. Besides, an age-related change showed in male cats that total L-carnitine level in geriatric cats was higher than kittens. Serum total L-carnitine concentration increased in cats with DM, neoplastic and cardiac disorders, while free L-carnitine concentration further increases in cats with cholangitis/hepatitis/pancreatitis, renal/urinary tract disorders and viral disorders, and acylcarnitine concentration further increased in GI disorders. Despite insulin resistance was considered to be involved with total and acylated L-carnitine elevation, hypercarnitinemia was also possibly the consequence of cellular L-carnitine release from damaged muscles under pathological conditions. The correlation of L-carnitine and haptoglobin might emphasize the role of L-carnitine in inflammation, especially hepatic disorders since both of them can be endogenously synthesized in the liver. Although haptoglobin has long been considered to be a moderate acute phase protein in cats based on the magnitude of the inflammatory response, the correlation between L-carnitine concentrations and haptoglobin level might highlight new perspectives on both of them. In conclusion, the potential of serum L-carnitine level as a positive disease marker in cats was supported.

Table1. Serum L-carnitine, SAA, haptoglobin and leptin concentrations in cats

Subgroup	Total carnitine (μM)	Free carnitine (μM)	Acylcarnitine (μM)	SAA (mg/l)	Haptoglobin (μg/ml)	Leptin (ng/ml)
Control	23.9±7.6 (n = 41)	20.0±6.4 (n = 41)	4.0±1.8 (n = 41)	0.07±0.08 (n = 18)	1307±1409 (n = 16)	38.35±16.73 (n = 18)
Diabetes mellitus	37.6±19.2* (n = 18)	30.2±16.4* (n = 18)	6.8±3.3** (n = 18)	16.96±48.91 (n = 18)	5404±7186* (n = 17)	13.85±8.55*** (n = 10)
Cholangitis/Hepatitis/Pancreatitis	53.8±51.2 (n = 12)	45.2±45.5* (n = 12)	8.7±7.2 (n = 12)	18.15±30.24* (n = 11)	4251±7537 (n = 12)	17.08±7.64*** (n = 11)
Renal/Urinary tract disorder	58.4±65.3 (n = 8)	48.6±54.3* (n = 8)	9.8±11.2 (n = 8)	0.88±1.83 (n = 8)	1847±2286 (n = 7)	14.65±8.05*** (n = 6)
Neoplastic disorder	39.2±32.3* (n = 39)	31.5±26.3** (n = 39)	7.7±7.1*** (n = 39)	10.56±19.10* (n = 39)	6326±6771** (n = 39)	19.50±5.96*** (n = 26)
GI disorder	28.4±13.5 (n = 11)	22.5±10.6 (n = 11)	5.9±4.4* (n = 11)	26.50±58.29 (n = 11)	3743±3547* (n = 11)	12.24±9.85*** (n = 7)
Viral disorder	47.0±55.0 (n = 10)	39.9±49.8* (n = 10)	7.1±6.4 (n = 10)	31.07±54.97* (n = 10)	9595±8755** (n = 10)	21.39±3.0** (n = 6)
Cardiac disorder	46.0±30.6** (n = 8)	37.9±28.0*** (n = 8)	8.2±3.7* (n = 8)	27.93±51.67* (n = 8)	5947±10487 (n = 8)	15.63±9.64** (n = 4)
Hematological disorder	27.8±13.1 (n = 16)	23.0±12.8 (n = 16)	4.8±1.3 (n = 16)	2.44±8.14 (n = 16)	4075±7176 (n = 15)	12.76±10.38*** (n = 7)
Others	22.7±8.9 (n = 17)	18.2±7.7 (n = 17)	4.5±2.0 (n = 17)	5.27±11.45 (n = 17)	3279±2294 (n = 17)	24.67±15.48** (n = 15)

1. Data above were showed as Mean±SD, n = sample number
2. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, significant elevation compared with control group

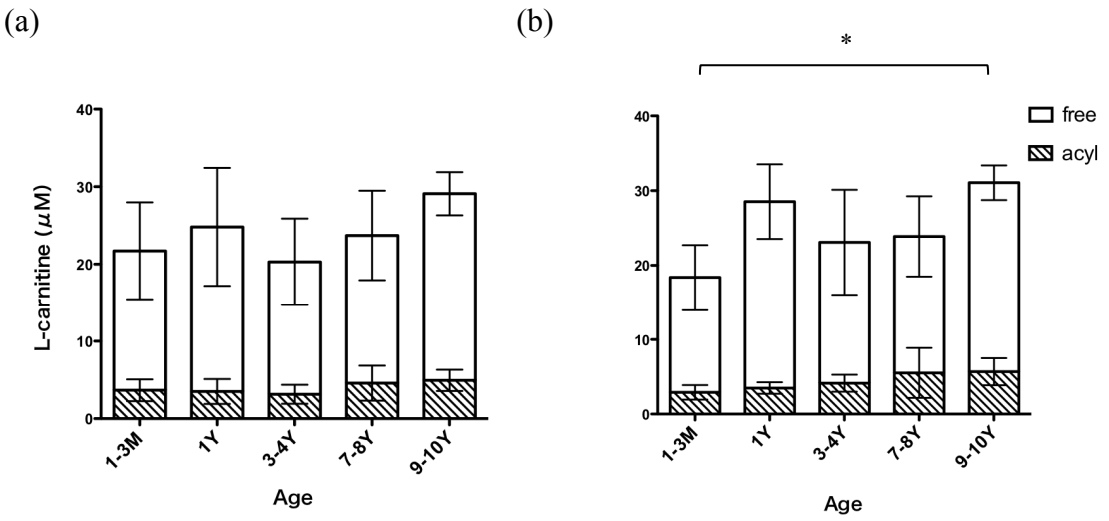
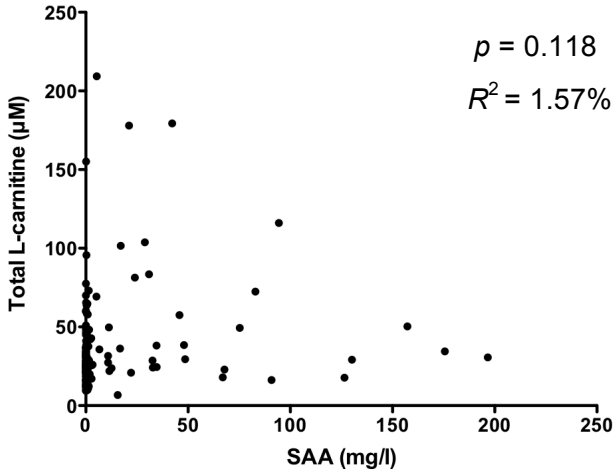


Fig. 1. Serum L-carnitine concentrations at different age. (a) shows serum L-carnitine levels in all cats and (b) shows L-carnitine concentrations in male cats. A positive correlation between acylcarnitine concentration and age was found in (a) (p=o.o458, R^2=9.84%). (b) shows serum L-carnitine levels in male cats. Total L-carnitine level in geriatric cats over 9 years of age was significantly higher than kittens under 3 months of age ($p<0.05$).

(a)



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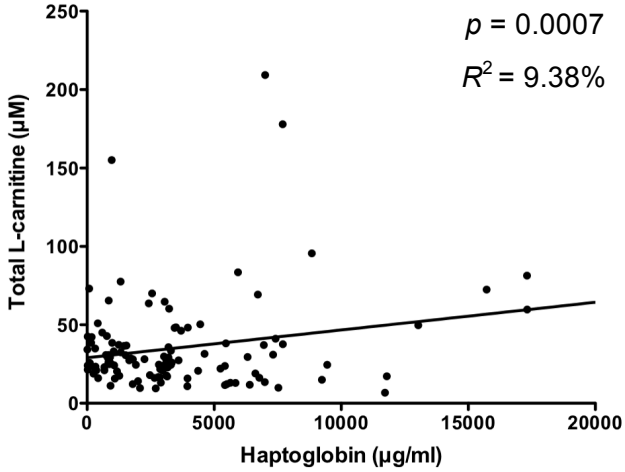


(c)

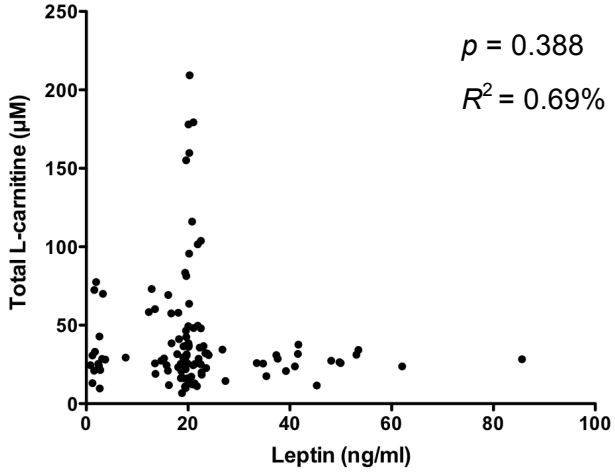


Fig. 2. Correlation of serum total L-carnitine and SAA (a), haptoglobin (b) and leptin (c). Also p = 0.0005, R^2 = 9.77% between haptoglobin and free L-carnitine, and p = 0.024, R^2 = 4.24% between haptoglobin and acylcarnitine (figures not shown).

Chapter II

An in vitro study on the efflux of L-carnitine from murine and feline skeletal myotubes under oxidative or hypoxic stress

Summary

Disease-associated serum L-carnitine elevation was considered to be the consequence of L-carnitine release from the skeletal muscles. To simulate the L-carnitine release from the skeletal muscle under oxidative and/or hypoxic conditions, *in vitro* models were employed. Mouse C_2C_{12} myoblasts and commercially available primary cultures of feline skeletal myocytes were used for this aim. These cells were differentiated into myotubes and pre-cultured in culture media supplemented with 250 mM L-carnitine to construct the intracellular L-carnitine pools. Cellular L-carnitine uptake was blocked by OCTN2 inhibitors (amiodarone, carvedilol, propantheline and verapamil), indicating skeletal muscle cells well presented OCTN2. After that, murine and feline myotubes were cultured under an oxidative model (cells cultured in the presence of 0.5 mM H_2O_2 for 12 hours) or a hypoxic model (cells cultured under 100% nitrogen atmosphere for 12 hours), and the intra- and extra-cellular L-carnitine amount was measured. Both in the oxidative and hypoxic models, release of L-carnitine into the extracellular space was accelerated. Additionally the OCTN2 inhibitors had no inhibitory effect on the L-carnitine release from the myotubes. In this Chapter, the disease-related release of L-carnitine from the skeletal muscle was reproduced at least in part *in vitro*, however, the efflux pathway of L-carnitine could not be identified. Intramyocellular L-carnitine might leak *via* passive permeability and/or unknown route.

2.1. Introduction

In previous chapter, disease-associated hypercarnitinemia was noticed in cats. Since L-carnitine concentrations in body tissues are generally 20 to 50 fold higher than in the plasma [88], and only a small fraction, approximately 0.1%, of total body L-carnitine is located within the blood, serum L-carnitine concentrations are supposed to be varying and easily influenced by L-carnitine released from other tissues. Similar L-carnitine elevation has also been noticed in human patients with heart failure and cancer [110, 119], and a negative correlation is found between blood and muscle L-carnitine concentrations additionally [105, 110]. Therefore, disease-associated L-carnitine level change is very likely to be the consequence of L-carnitine efflux from the skeletal muscle, the largest L-carnitine reservoir in the body.

Oxidative stress is related with a variety of diseases including diabetes, cancer and cardiovascular diseases [83]. Also disease-associated serum L-carnitine elevation was found in cats with DM, neoplastic and cardiac disorders in the previous chapter. So oxidative stress *in vitro* might mimic pathological conditions associated with hypercarnitinemia. The ROS hydrogen peroxide (H_2O_2) can be naturally produced by oxygen free radicals such as superoxide. Although H_2O_2 is a weak oxidizing and reducing agent, it can further form much more damaging species like hydroxyl radicals by reacting with iron [41]. H_2O_2 is commonly used in laboratories to induce oxidative stress and is proved to cause mitochondrial fragmentation, stimulate protein catabolism in C_2C_{12} cell with non-cytotoxic dose [29, 62]. In addition, hypoxic stimulus can influence the skeletal muscle in several ways including affect oxidative enzymes and formation of ROS [17].

For decades, studies focus on L-carnitine uptake ability into cells and the effect of supplementation for L-carnitine deficiency is considered to be an important issue in human medicine. Treatment of triiodothyronine and insulin are reported to increase L-carnitine accumulation in skeletal muscles while iron decreases L-carnitine uptake activity [60, 107]. However, there is a lack of research about the mechanism of cellular L-carnitine release. Even though OCTN2 is proved to hold the primary role in transporting L-carnitine into the cells, the mechanism of cellular L-carnitine release is still not established[88]. Yet the mechanism of cellular L-carnitine loss by diffusion or facilitated export to adjust tissue L-carnitine concentration is believed to exist [86]. In vitro approach of OCTN2 inhibitors is proved to reduce L-carnitine uptake in cultured cells [24], but it is undetermined if OCTN2 is also involved in cellular L-carnitine release or not. Since OCTN2 is the major L-carnitine transporter besides passive diffusion [88], the possibility that OCTN2 is also associated with cellular L-carnitine release can't be ignored.

The aim of this study is to investigate dynamic L-carnitine concentration change in skeletal muscle cells under oxidative and hypoxic stress, also to understand the role of OCTN2 in L-carnitine release by application of OCTN2 inhibitors.

2.2. Materials and Methods

Cell preparation and whole cell extraction

To invest skeletal muscle L-carnitine dynamic under pathological conditions, an established model of skeletal muscle: mouse myoblast C_2C_{12} cell line was gifted from Laboratory of Veterinary Physiology, the University of Tokyo. Also feline skeletal muscle cells (FSkMC) isolated from the limbal skeletal muscle were purchased from Cell Applications Inc. (San Diego, USA). C_2C_{12} cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Inc., St Louis, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences Inc., St Louis, USA) and 0.5 mg/ml gentamycin (Nippon Zenyaku Kogyo Co., Ltd. Fukushima, Japan) and incubated at 37°C in a water saturated atmosphere of 95% ambient air and 5% CO_2. Myogenic differentiation of confluent C_2C_{12} cells into myotubes was induced by replacing culture media into DMEM supplemented with 2% FBS and antibiotics. FSkMC were grown in Feline Skeletal Muscle Cell Growth Medium (Cell Applications) and differentiated in DMEM supplemented with 10% FBS and 0.5 mg/ml gentamycin under 95% air and 5% CO_2 at 37.0°C. In the present study, skeletal muscle cells were cultured on 25 mm round sterile plastic cover slides (Thermo Fisher Scientific Inc., Rochester, USA) into monolayers over 90% confluence and then differentiated for 2 weeks in differentiation media. Cell fusion of skeletal muscle cells into myotubes was observed microscopically and considered to be ready for treatment after differentiation rate reached 70%. To measure intracellular L-carnitine concentration, whole cell extraction was performed. Myotubes on 1 cover slide was acidified with 500 µl 0.6 M $HClO_4$ at first, and then neutralized by 150 µl 2.0 M

K_2CO_3. After centrifuged to remove insoluble salts, L-carnitine concentration in the supernatant was measured with Total Carnitine Assay Kit (Kainos Laboratories, Inc., Tokyo, Japan) using a Model 7180 automated clinical chemistry analyzer as described in chapter I (Hitachi High-Technologies, Tokyo, Japan).

The ability of L-carnitine uptake in C_2C_{12} myotubes

Differentiated C_2C_{12} myotubes were cultured in the presence of 10, 25, 100, 250 or 500 μM L-carnitine hydrochloride (Sigma-Aldrich) in DMEM supplemented with 2% FBS and antibiotics for 48 hours. Intracellular total L-carnitine concentration was measured to evaluate the ability of L-carnitine uptake as described above.

Intracellular L-carnitine concentrations under oxidative or hypoxic stress

C_2C_{12} and FSkMC myotubes were pretreated with 250 μM L-carnitine for 24 hours, and then separately cultured in H_2O_2 group or low O_2 group. In H_2O_2 group, myotubes were cultured in 0.5 mM H_2O_2 (Wako Pure Chemical, Osaka, Japan) supplemented differentiation media as oxidative stress, and in low O_2 group, myotubes were cultured in differentiation media inside a nitrogen chamber as hypoxic stress. Intracellular L-carnitine concentration was measured at 24 and 48 hours of treatment. In C_2C_{12} group, trypan blue cell exclusion test was performed to evaluate cell viability and L-carnitine level of culture media as extracellular L-carnitine concentration was also measured.

Effect of OCTN2 inhibitor on cellular L-carnitine uptake and release

OCTN2 inhibitors including amiodarone hydrochloride, propantheline bromide (MP Biomedicals, LLC, Illkirch, France), carvedilol (LKT Laboratories, Inc., St Paul, US) and verapamil hydrochloride (Wako) were all tested on C_2C_{12} myotubes while

only less-cytotoxic amiodarone and propantheline were tested on FSkMC. To test effect of OCTN2 inhibitors on cellular L-carnitine uptake, myotubes were cultured in differentiation media supplemented with 250 μM L-carnitine and OCTN2 inhibitors in 3 different concentrations for 12 hours. For each drug had varied cytotoxicity, tested concentrations of amiodarone and propantheline were 0.5, 1.0 and 2.0 mM and concentrations of carvedilol and verapamil were 50, 100 and 250 μM on C_2C_{12} myotubes. Only 0.5 mM amiodarone and propantheline were tested on FSkMC. To observe influence of OCTN2 inhibitors on cellular L-carnitine release, myotubes were pretreated with 250 μM L-carnitine for 24 hours, and then cultured in differentiation media supplemented with OCTN2 inhibitors for 12 hours. To reach maximum but non-cytotoxic concentration, 1 mM amiodarone, 1 mM propantheline, 250 μM carvedilol and 500 μM verapamil were tested on C_2C_{12} cells, and 0.5 mM amiodarone and 0.5 mM propantheline were applied on FSkMC.

Statistic analysis

Comparisons between control and experiment groups were analyzed by Mann-whitney test. Correlation of L-carnitine concentrations between cultured cells and medium were analyzed by Pearson's correlation. GraphPad Prism for Macintosh OS ver. 5 was used and obtained *p* value under 0.05 was considered to be statistically significant.

2.3. Results

The ability of L-carnitine uptake in C_2C_{12} myotubes

L-carnitine uptake ability of C_2C_{12} myotubes was shown in Figure 1. It was found, at least under the environmental concentration of 500 μM L-carnitine, skeletal muscle cell L-carnitine concentration was proportional to culture media with a strongly positive correlation between intracellular and extracellular L-carnitine concentrations was confirmed.

Intracellular and extracellular L-carnitine concentrations under oxidative or hypoxic stress

Significant intracellular L-carnitine decrease and extracellular L-carnitine increase under oxidative or hypoxic stress were observed in C_2C_{12} cells, also similar intracellular L-carnitine losing under stress was noted in FSkMC myotubes (Fig 3). Cultured myotubes showed spontaneous L-carnitine efflux in L-carnitine-free environment, and it was observed that the L-carnitine release was more immense in FSkMC than in C_2C_{12} cells. After treatment of 0.5 mM H_2O_2 or low O_2 conditions, significant intracellular L-carnitine decrease with extracellular L-carnitine increase was found, while the cell viability over time was never under 94% in C_2C_{12} myotubes (Fig 2), indicating cellular L-carnitine release from skeletal muscles under oxidative or hypoxic stress with limited influence from cell apoptosis or cell membrane rupture.

Effect of OCTN2 inhibitor on cellular L-carnitine uptake and release

Inhibition of L-carnitine uptake in skeletal muscle cells by OCTN2 inhibitors was showed in Figure 3. All tested drugs including amidarone, propantheline, carvedilol and verapamil induced significant L-carnitine uptake inhibition, but the inhibition was not dose-dependent with the concentrations performed in current study.

Furthermore, treatment with OCTN2 inhibitors accelerated cellular L-carnitine release significantly (Fig 4). The blockage of OCTN2 transporter didn't stop L-carnitine release, suggesting that indicating L-carnitine release isn't via OCTN2.

2.4. Discussion

This *in vitro* study supported the mechanism proposed by Vescovo that plasma L-carnitine elevation is possibly the consequence of muscular L-carnitine release under pathological conditions [119]. In the previous chapter, serum L-carnitine elevation is noticed in several disease conditions including DM, neoplastic and cardiac disorders, also one of the most potential causes of hypercarnitinemia is cellular L-carnitine release from the skeletal muscle. In this chapter, L-carnitine release from both murine and feline skeletal muscle cells was noticed and assumed to cause disease-associated serum L-carnitine level change in cats. The efflux of L-carnitine from human heart cell line due to accelerated exchange diffusion was reported [74]. However, there was no further research reported thus the underlying mechanism of cellular L-carnitine release remained unknown.

Blocking OCTN2 transportation by its inhibitors accelerated intramyocellular L-carnitine releasing. In other words, OCTN2 inhibition couldn't stop L-carnitine efflux but cut off the continuous L-carnitine uptake, indicating L-carnitine uptake by OCTN2 was the major method to maintain intracellular L-carnitine concentrations in the skeletal muscle. The impact of OCTN2 inhibitors on L-carnitine releasing in the skeletal muscle was notable, leading to the concern of prescribing drugs with OCTN2 inhibition effect. Amiodarone was a generally used antiarrhythmic agent, reported to attenuate ischemia-induced fatty acid accumulation in feline cardiac tissues [98]. Since cellular L-carnitine retention was supposed to reduce after treatment of amiodarone, decreased feline cardiac tissue fatty acids content might accompany with reduction of L-carnitine level for decreased fatty acids metabolism. Propantheline, a quartenary

ammonium antimuscarinic agent used in feline idiopathic cystitis for treating incontinence, and verapamil, a calcium channel blocker, were reported to evoked muscular weakness and adynamia in cats [6], possibly also related to muscular L-carnitine lose. In addition, carvedilol reduced the contractile response of diaphragm to nerve or muscle stimulation [43]. It's intriguing that OCTN2 can be inhibited by multiple agents those are responsible for different mechanisms. There are much more clinically used OCTN2 inhibitors for varied purposes, mostly cardiac prescription drugs [39, 90], the long-term use should be cautious and L-carnitine monitor might be necessary for the possibility of systemic L-carnitine losing and decrease whole body L-carnitine retention.

Despite facilitated export of intracellular L-carnitine is supposed to exist, there hasn't been any transporter known for in charge of cellular L-carnitine release except for passive diffusion [86, 117, 124]. In light of the efflux pathway of L-carnitine could not be identified, intramyocellular L-carnitine might leak *via* passive permeability and/or unknown route. Activation of transcriptional factors involved in gene regulation was found in C_2C_{12} cells after exposure to H_2O_2 at concentrations that did not alter cell viability [84]. Also gene expression could be altered in response to oxidative stress and the transcript level was correlated with the level of oxidative damage to the cells [32]. The influence of hypoxia on gene expression and mRNA level *in vitro* was also reported [4, 93]. Taken together, intramyocellular L-carnitine efflux might be involved with transcriptional regulators, and further research is needed to interpret the mechanism under cellular L-carnitine release from the skeletal muscle.

2.5. Conclusion

The efflux of L-carnitine from the skeletal muscle was confirmed through *in vitro* studies performed in murine and feline skeletal muscle cells. Intramyocellular L-carnitine released significantly under oxidative or hypoxic stress, suggesting hypercarnitinemia under pathological conditions in diseased cats was possibly the consequence of L-carnitine losing from skeletal muscles. Also the blockage of cellular L-carnitine transporter OCTN2 accelerated L-carnitine losing, indicating L-carnitine release wasn't via OCTN2, however, the mechanism of transporting L-carnitine outside cells was still unclarified.

2.6. Figures

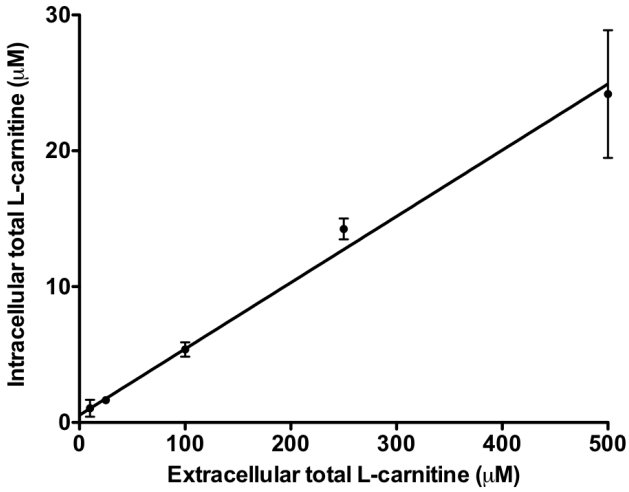


Fig. 1. Concentrations of intracellular L-carnitine level after cultured in the media supplemented with different L-carnitine concentration.

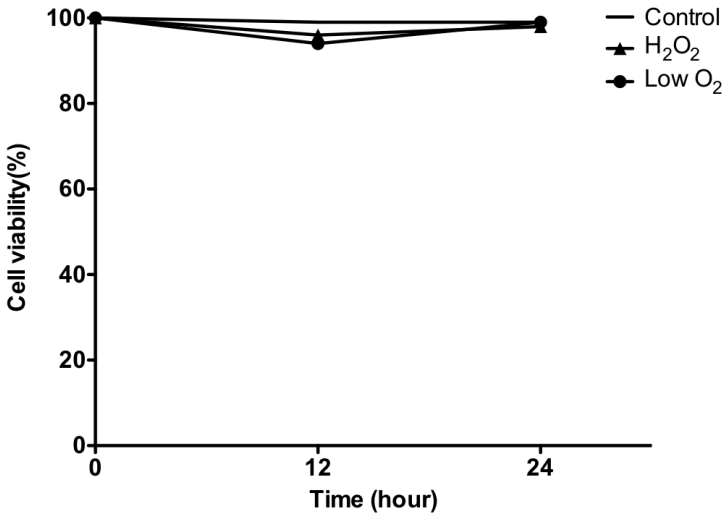


Fig. 2. Cell viability of C_2C_{12} cells in untreated cells (control), cells treated with oxidative stress (H_2O_2) and cells treated with hypoxic stress (Low O_2).

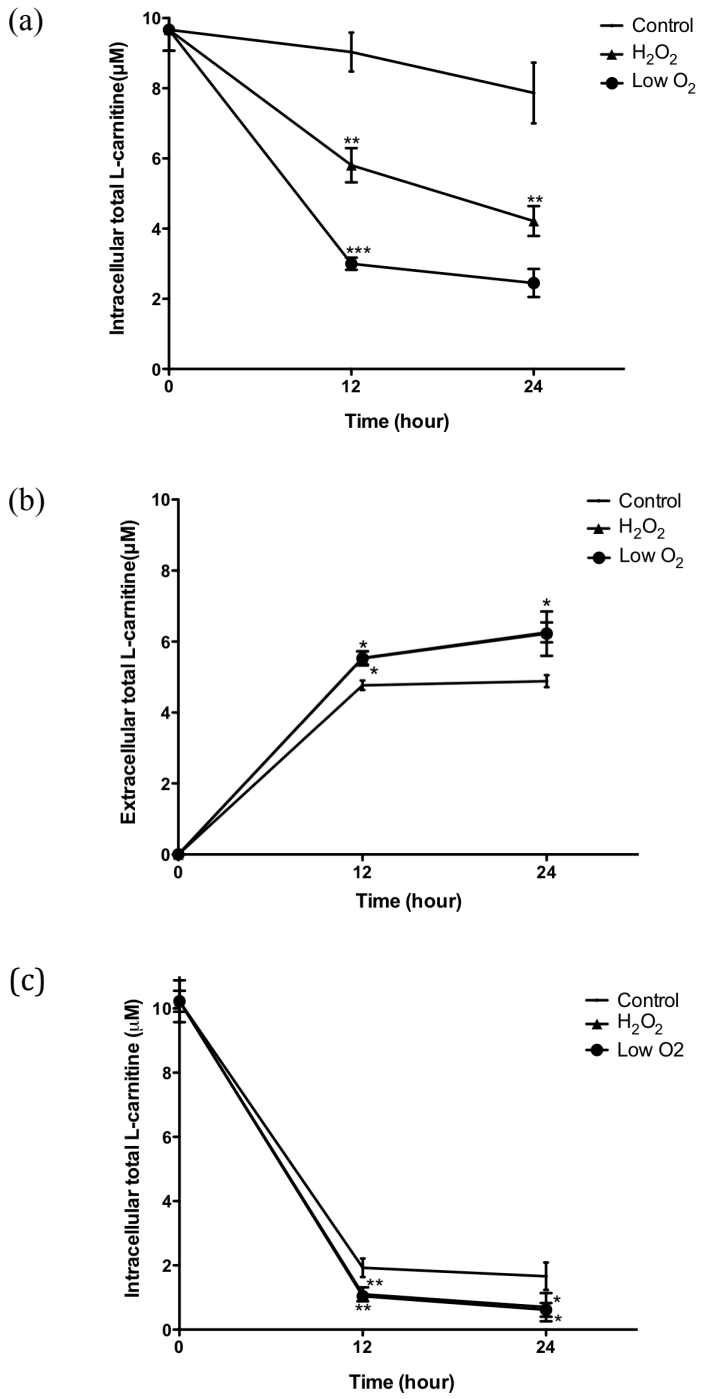


Fig. 3. Cellular L-carnitine release in C_2C_{12} cells and FSkMC. (a) and (b) shows intracellular and extracellular L-carnitine concentrations estimated in C_2C_{12} myotubes. After 12- and 24- hour treatment of H_2O_2 and low O_2, significant intracellular L-carnitine reduction and extracellular L-carnitine increase were noticed. For extreme cytotoxicity induced in low O_2 group, sample numbers at 24 hour was too limited to get statistical significance. (c) shows intracellular L-carnitine concentration change in FSkMC, significant L-carnitine reduction was also observed

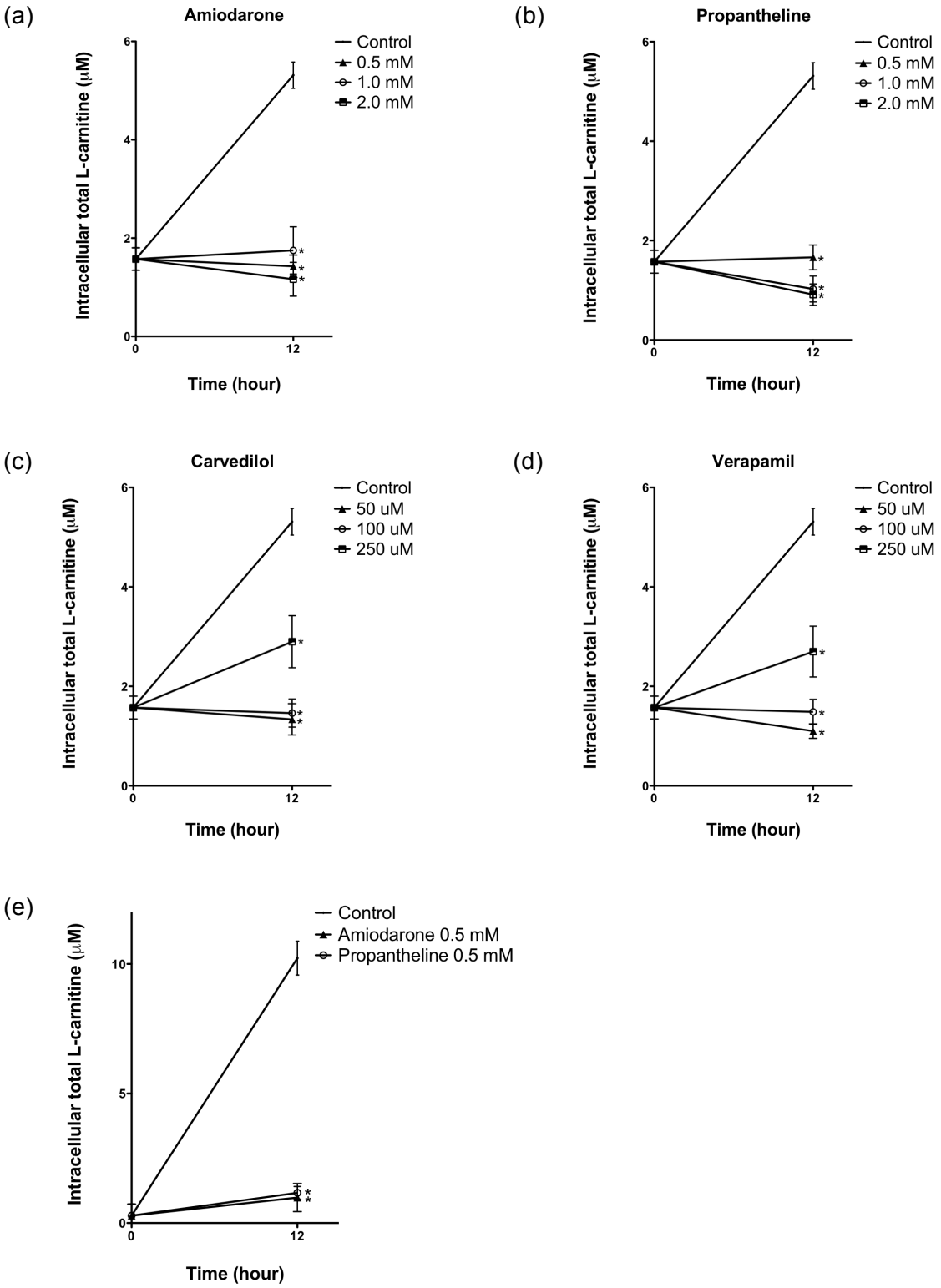
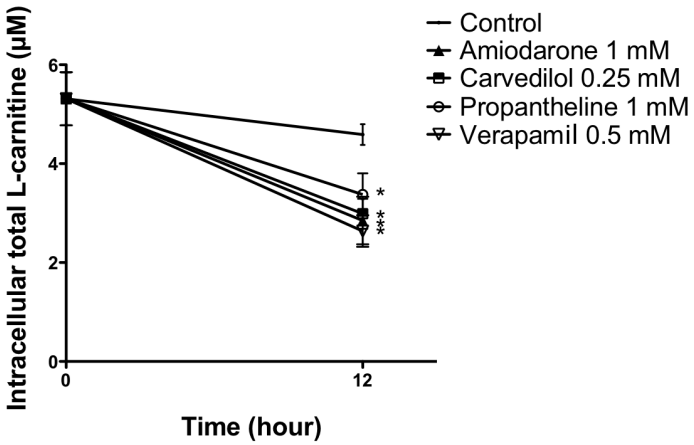


Fig. 3. Effect of L-carnitine uptake inhibition by OCTN2 inhibitors. 12-hour treatment of amiodarone (a) and propantheline (b) at 0.5, 1.0 and 2.0 mM and carvedilol (c) and verapamil (d) at 50, 100 and 250 μM successfully antagonized L-carnitine uptake in C_2C_{12} cells ($p < 0.05$). Similar L-carnitine uptake inhibition was also found in FSkMC with 0.5 mM amiodarone and propantheline (e).

(a)



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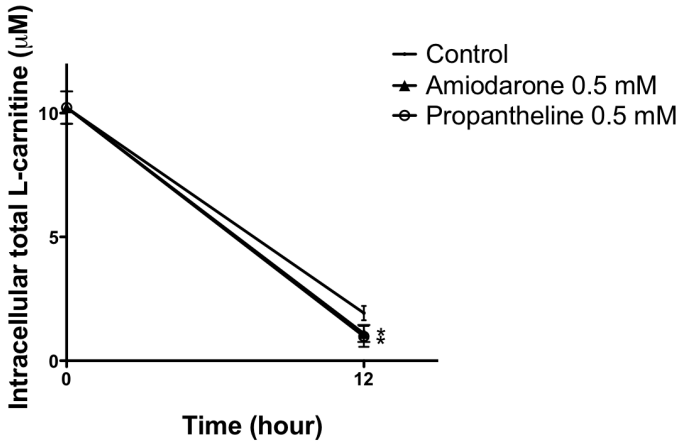

Fig. 4. Influence of OCTN2 inhibitors on L-carnitine pretreated skeletal muscle cells. Acceleration of L-carnitine release caused by 1 mM amiodarone, 1 mM papropantheline, 250 μM carvedilol and 500 μM verapamil was found in C_2C_{12} myotubes (a) ($p < 0.05$). Similar accelerated L-carnitine release caused by 0.5 mM amiodarone and 0.5 mM papropantheline was also found in FSkMC (b) ($p < 0.05$).

Chapter III

In vitro genetic analysis of L-carnitine release mechanism from murine C_2C_{12} myotubes under oxidative and hypoxic stress

Summary

It's known that cellular L-carnitine uptake is via its transporter OCTN2. Cellular L-carnitine release was observed in murine skeletal muscle C_2C_{12} myotubes under oxidative stress caused by 0.5 mM H_2O_2 supplementation and hypoxic stress caused by low O_2 environment. However, the mechanism under muscular L-carnitine efflux remains indeterminate. Microarray technology might help to reveal the underlying transcriptional regulatory factors. In this chapter, affymatrix GeneChip was used to analyze the gene expression of C_2C_{12} myotubes under oxidative or hypoxic stress. The result showed 191 probe sets differentially expressed in H_2O_2 treated group and 225 probe sets differentially expressed in low O_2-treated group. Gene expression of *Mrpl52, Fryl, Mtnd1* and *Psph* increased significantly and gene expression of *RNF220* and *CtBP1* decreased significantly under oxidative stress. Significantly up-regulated expression of *RFK*, *Mxi1*, *Ero1l*, *Psph* and *Mrpl52*, also significantly down-regulated expression of *RNF220* and *mCLOCK* were found under hypoxic stress. Significant up-regulation of *Mrpl52* and down-regulation of *RNF220* were found in both oxidative and hypoxic stress groups, indicating the increase of mitochondrial transcripts and suppression of ubiquitin proteasome system (UPS) might accompany with intramyocellular L-carnitine release.

3.1. Introduction

Intramyocellular L-carnitine release under pathological conditions such as oxidative or hypoxic stress is considered to result in hypercarnitinemia in diseased cats according to our previous chapters. However, the mechanism underlying the release of L-carnitine from skeletal muscles remains indeterminate. The OCTN family, especially OCTN2, is responsible for regulating membrane transport of L-carnitine into the cells in mice and humans [112]. Although in former chapter, OCTN2 inhibition didn't prevent intracellular L-carnitine efflux, indicating that L-carnitine release isn't via OCTN2, but an involvement of a transporter belonging to the same superfamily still can't be excluded.

On the other hand, inhibition of CPT1, the enzyme that transfers cytoplasmic L-carnitine to acylcarnitine, which can then translocate into mitochondria, and down-regulated peroxisome PPARα expression are caused by increased ROS production in C_2C_{12} myotubes [10]. Besides, the incubation of murine C_2C_{12} skeletal muscle cells with H_2O_2 is reported to stimulate protein degradation and ubiquitin-conjugating activity [37, 62]. Therefore the acceleration of cellular L-carnitine release under oxidative stress induced by H_2O_2 or hypoxic stress induced by low O_2 environment might be explained through the transcript levels of genes.

Microarray technology has been used as an efficient and reliable method to study the gene expression and sequence variation over the last few decades and GeneChip microarray is a further standardized system as a single platform [22]. Microarrays have been useful to analyze transcriptomic differences from tissue biopsies or cultured muscle cells [38, 59, 63, 120]. However, feline microarray is not

available yet while human microarrays are used for feline transcriptional analysis so far [18, 25]. Although there is over 85% similarity in human and feline gene sequence [18, 25], in order to obtain accurate gene expression data, mouse skeletal muscle C_2C_{12} myoblast was chosen in this study to understand the mechanism of L-carnitine release from skeletal muscles for similar L-carnitine efflux was observed in both murine and feline skeletal myotubes under oxidative or hypoxic stress.

3.2. Materials and Methods

Cell preparation and treatment

C_2C_{12} myotubes preparation and treatment was performed as described in chapter II. In short, C_2C_{12} myoblasts incubated in 9-cm diameter petri dishes at 37°C in a water saturated atmosphere of 95% ambient air and 5% CO_2 were cultured in DMEM supplemented with 10% FBS and 0.5 mg/ml gentamycin and then differentiated into myotubes in DMEM supplemented with 2% FBS and antibiotics. After 2 weeks of differentiation, 24-hour treatment of H_2O_2 or low O_2 was applied. 0.5 mM H_2O_2 was added into culture media to produce oxidative stress while incubation within a nitrogen chamber as low O_2 environment was considered to cause hypoxic stress.

RNA isolation

Total cellular RNA was extracted from oxidative stress-treated, hypoxic stress-treated and untreated mouse C_2C_{12} myotubes using RNeasy Mini Kit (Qiagen Sciences, Maryland, USA) followed instruction manual. RNA concentration was determined by absorption at 260 nm wavelength with a V-630 Bio spectrophotometer (JASCO Ltd., Essex, UK), and sample quality was verified using the equation that the absorption at 260 nm should be about twice the absorption at 280 nm. Prepared RNA samples were stored at –140°C.

Microarray hybridization

Gene expression of 6 skeletal muscle myotube samples was analyzed by GeneChip® Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, USA) representing 28,853 genes. All the samples including oxidative stress-treated, hypoxic stress-treated

and normal mouse C_2C_{12} myotubes were analyzed in dublicate. Microarrays were processed according to Affymetrix protocols (GeneChip expression analysis, Technical manual from Affymetrix). Obtained data was processed by AltAnalyze (Gladstone Institutes, University of California at San Francisco, USA). Fold change of gene expression $\geqq$ 2.0 and $\leqq$ -2.0 was considered differentially expressed and fold change expression $\geqq$ 4.0 and $\leqq$ -4.0 was considered significantly expressed.

3.3. Results

A total of 191 probe sets were differentially expressed in H_2O_2-treated group (90 up-regulated and 101 down-regulated), and 225 probe sets were differentially expressed in low O_2 treated group (102 up-regulated and 123 down-regulated). There were 57 differentially expressed sequences overlapped in both groups, comprising 10 up-regulated genes and 11 down-regulated genes (22 up-regulated sequences and 35 down-regulated sequences).

The most strongly up- or down-regulated genes in skeletal muscle myotubes under oxidative or hypoxic stress were showed in Table 1. Gene expression of *Mrpl52, Fryl, Mtnd1* and *Psph* increased significantly and gene expression of *RNF220* and *CtBP1* decreased significantly under oxidative stress caused by treatment of H_2O_2. Hypoxia stress significantly up-regulated expression of *RFK*, *Mxi1*, *Ero1* and *Mrpl52*, also significantly down-regulated expression of *RNF220* and *mCLOCK*. Above all genes, enhanced *Mrpl52* and suppressed *RNF220* were significant under both oxidative and hypoxic stress. Moreover, differentially up-regulation of *Arfgef1, Ddb1, Hspd1, Mtnd1, TrpRS* and *Vta1*, and differentially down-regulation of *Abhd16a, Atp6v0d1, Dscr3, Golm1, Hd5, Hp1bp3, Psma1, Rab14* and *Ythdf1* were found in both groups.

L-carnitine-related gene expression was listed in Table 2. There was no obvious effect of oxidative or hypoxic stress on L-carnitine-related gene expression including genes involved in L-carnitine and the OCTN family, but these treatment slightly up-regulated important L-carnitine transportation-related genes including CPT1, CPT2, CACT and OCTN2.

Table 3 showed the gene expression data involved in insulin signaling, UPS and muscle wasting, which was supposed to associate with L-carnitine function. Differentially differences in oxidative or hypoxic groups were noticed in 10 UPS-related genes but only *Psma1* overlapped in both groups.

3.4. Discussion

In current study, oxidative and hypoxic stress shared similar influence on gene expression of *Mrpl52* and *RNF220* significantly, also there was differential expression of a variety of genes in both groups. However, the specific L-carnitine-related genes supposed to be involved in cellular L-carnitine release was not observed. The difficulty of interpreting the results of microarray analysis is that sometimes the most expressed sequences belong to unknown genes, and also there is more than 1 probe set for 1 gene because the attached sequences on the gene chip are separated pieces of a whole length genome. So it's not rare to get confusing result including *Anapc1*, *Atp6v0d1*, *Mtif2*, *TrpRS*, and *Xpo7* with both differentially up-regulated and down-regulated sequences.

Mitochondria precursor *Mrpl52* represents expression of the large unit of the mammalian mitochondria ribosome while *Mtnd1* is a mitochondrial membrane NADH dehydrogenase subunit, playing a part in the electron transport chain of oxidative phosphorylation associated with mitochondrial transcript levels [56, 123]. Both of them associated with mitochondrial transcript levels. Up-regulation of *Mrpl52* might indicate activated mitochondria protein synthesis for the mitochondrial translational system subunit *Mtnd1* was also highly expressed, but its function was still unclear [16, 85]. Since the mutation in *Mtnd1* is involved with oxidative stress-related mitochondrial disorders in human medicine [123], the possible explanation of altered expression of *Mtnd1* accompanied with up-regulated *Hspd1* in this study was the oxidative stress in both groups.

RNF220 was an E3 ubiquitin ligase while *Psma1* was the subunit of proteasome [45, 58], both belonged to UPS, a cellular degradation system playing an important role

in maintaining normal cellular function [95]. Therefore significant repressed expression of UPS-related genes was noticed to accompany with cellular L-carnitine release caused by treatment of oxidative and hypoxic stress. UPS and mitochondria ribosome involved in maintaining protein homeostasis under conditions such as oxidative stress [95], and the failure or malfunction of protein homeostasis network was often associated with diseases [64]. Stimulation of H_2O_2 up-regulated ubiquitin-conjugating enzyme activity in skeletal muscle cells was reported, indicating UPS involved in muscle wasting under pathological conditions [37, 62]. In addition, the beneficial effects on skeletal muscle cells caused by L-carnitine supplementation was through down-regulation of UPS-related genes in rats and piglets, probably via modulating the inhibitor of UPS [53, 54, 55]. However, we found an opposite result that suppressed UPS gene expression was accompanied with disease-associated muscular L-carnitine release. Still we can't rule out the possibility that the influenced genes in both groups merely caused by mutual oxidation caused by H_2O_2 or low O_2. Taken together, these findings lead to the unclarified role of UPS in L-carnitine releasing mechanism.

Insulin resistance was considered to be relevant to hypercarnitinemia described in chapter I. Additionally, insulin was validated to increase L-carnitine accumulation in skeletal muscle cells and stimulate plasma L-carnitine clearance [107, 108], indirectly suggesting insulin resistance might lead to circulating L-carnitine elevation. However in current study, no differential expression was found in genes involved in insulin signaling including *MAPK*, *PI3K*, *FOXO3* and *PGC-1* [121], suggesting that circulating L-carnitine level and insulin resistance might be irrelevant. On the other

hand, muscle damage under pathological conditions was presumed to lead to myocellular L-carnitine release and hypercarnitinemia was proved to be the consequence of intramyocellular L-carnitine efflux [110, 119]. Still there wasn't obvious regulation noted in genes involved in muscle damage such as *Chrna1* or *Tfrc* [47, 65], suggesting cellular L-carnitine release was not led by muscle damage.

The Affymatrix feline GeneChip is available by now. For L-carnitine-related genetic expression seems to be more obvious in *in vivo* study compared with *in vitro* experiments [54], further research is expected to unveil the underlying mechanism of intramyocellular L-carnitine release.

3.5. Conclusion

There were 57 differentially expressed sequences overlapped in both groups, comprising 10 up-regulated genes and 11 down-regulated genes in mouse skeletal muscle C_2C_{12} myotubes under oxidative or hypoxic stress. The most expressed genes were significantly up-regulated *Mrpl52* and down-regulated *RNF220*, and differentially up-regulated *Mtnd1* and down-regulated of *Psma1*. These finding indicated intramyocellular L-carnitine releasing accompanied with increased mitochondrial transcripts and suppression of UPS. However, the specific genes related to cellular L-carnitine release were not observed, and the mechanism under intramyocellular L-carnitine efflux besides passive diffusion remained unclear.

3.6. Tables

Table. 1. The most strongly up- and down-regulated genes in C_2C_{12} myotubes treated with oxidative and hypoxic stress

Probe set	Gene symbol	Description	Fold change	
			Oxidative stress	Hypoxic stress
Up-regulated genes (top 20)				
10341316	Anapc1	Anaphase-promoting complex subunit 2 is a component of APC/C, a cell cycle-regulated E3 ubiquitin ligase that controls progression through mitosis by mediating ubiquitination and subsequent degradation of target proteins.	2.06	2.10
10343148			2.55	2.06
10339527	Arfgef1	ADP-ribosylation factor guanine nucleotide-exchange factor 1 plays an important role in intracellular vesicular trafficking and the maintenance of Golgi structure.	3.14	3.27
10339648	Atp6v0d1	V-type proton ATPase subunit d 1 is a subunit of vacuolar ATPase, providing the energy required for transport processes and playing a role in coupling of proton transport and ATP hydrolysis	2.10	2.10
10414269	Bnip3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, an apoptosis-inducing protein involved in the degradation of damaged proteins inside mitochondria.	-1.16	3.03
10343581	Ddb1	DNA damage-binding protein 1 is required for DNA repair and also appears to function as a component of numerous distinct DCX E3 complexes which mediate the ubiquitination and subsequent proteasomal degradation.	2.60	2.64
10419198	Ero1l	ERO1-like gene, a member of the endoplasmic reticulum oxidoreductin family, might play a role in endoplasmic reticulum stress-induced apoptosis and the cellular response to hypoxia.	1.57	4.76*
10341565	Fryl	Furry homolog-like isoform 1 plays a key role in maintaining the integrity of polarized cell extensions during morphogenesis.	5.71*	1.09
10354732	Hspd1	Heat shock protein 1 implicates in mitochondrial protein import and facilitates the correct folding of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.	2.34	2.42
10404053	Hist1h2bc	Histone cluster 1, H2bc, is core component of nucleosome playing a role in transcription regulation, DNA repair, DNA replication and chromosomal stability.	1.15	3.56
10341601	Mrpl52	39S ribosomal protein L52 is a component of the mitochondrial ribosome large subunit so is considered a mitochondrial precursor.	5.75*	4.08*
10341178	Mtif2	Translation initiation factor IF-2 is an essential component for the initiation of protein synthesis involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex.	2.01	2.19
10598032	Mtnd1	NADH-ubiquinone oxidoreductase chain 1 and 4 is core subunits of the mitochondrial membrane NADH dehydrogenase required for catalysis and oxidative phosphorylation.	5.09*	3.45
10598062	Mtnd4		3.80	-1.65
10468517	Mxi1	Max interacting protein 1 is a transcriptional repressor.	1.04	4.58*

10341889	U6	U6 spliceosomal RNA is the non-coding small nuclear RNA component of U6 snRNP that unmodified pre-mRNA and other proteins to assemble a spliceosome.	1.22	3.60
10341155	RFK	Riboflavin kinase catalyzes the phosphorylation of riboflavin to form FMN and is essential for TNF-induced reactive oxygen species (ROS) production	1.95	5.95*
10340917	Psph	Phosphoserine phosphotase(PSPase) catalyzes the last step in the biosynthesis of serine from carbohydrates.	4.31*	-1.00
10344003			3.66	1.26
10342531	TrpRS	Cytoplasmic tryptophanyl-tRNA synthetase associated with tryptophan and amino acid metabolism	3.01	3.08
10338768	Vta1	Vacuolar protein sorting-associated protein involves in the endosomal multivesicular bodies (MVB) pathway delivering to lysosomes enabling degradation of membrane proteins.	2.10	2.00
10344566			3.03	1.04
10342331	Xpo7	Exportin-7 binds to the GTPase and mediates the nuclear export of proteins.	2.40	2.58
10344481			2.20	3.50
Down-regulated genes (top 20)				
10338817	Abhd16a	Abhydrolase domain-containing protein 16A might involve in hydrolase activity.	-2.32	-2.16
10342423	Anapc1	Anaphase-promoting complex subunit 2	-2.27	-2.01
10342775	Atp6v0d1	V-type proton ATPase subunit d 1	-2.77	-2.54
10342488	Ca091	UPF0546 membrane protein C1orf91 homolog is a mulyi-pass membrance protein.	-1.52	-3.63
10338831	CtBP1	C-terminal-binding protein 1 involves in controlling the equilibrium in the Golgi complex, functions in brown adipose tissue differentiation and has dehydrogenase activity.	-2.91	-1.44
10342637			-4.17*	-1.33
10338192	Dscr3	Down syndrome critical region protein 3 homologe	-2.10	-2.16
10338464			-2.99	-2.34
10339491	Golm1	Golgi membrane protein 1 indicates cellular response to viral infection.	-2.69	-2.44
10339449	Hd5	Histone deacetylase 5, responsible for the deacetylation of lysine residues, involves in muscle maturation. During muscle differentiation, it shuttles into the cytoplasm, allowing the expression of myocyte enhancer factors.	-2.76	-2.10
10344327			-2.10	-2.24
10343097			-2.54	-2.37
10339374	Hp1bp3	Heterochromatin protein 1-binding protein 3 might involve in chromatin structure and function.	-2.11	-2.08
10338933	mCLOCK	Circadian locomoter output cycles protein kaput has intrinsic histone acetyltransferase activity and plays a role in DNA damage response.	1.34	-2.97
10343616			1.07	-4.06*
10342578	Mtif2	Translation initiation factor IF-2	-2.11	-2.09
10484735	Olfr1175	Olfactory receptor 1175 is one of olfactory receptors that interact with odorant molecules to initiate the perception of a smell.	-2.95	1.11
10343666	Psma1	Proteasome subunit alpha type-1 has an ATP-dependent proteolytic activity, mediates proteasome degradation.	-3.02	-2.67
10342433	Rab7a	Ras-related protein is key regulator in endo-lysosomal trafficking and also plays a central role in many other cellular events including nutrient-transportor mediated nutrient uptake and lipid metabolism.	-1.79	-3.30
10343132	Rab14		-2.04	-2.51
10340970	RNF220	RING finger protein 220 is an E3 ubiquitin-protein ligase that promotes the ubiquitination and proteasomal degradation	-5.64*	-4.20*
10344588			-3.63	-2.42

10340947	Rpl23	60S ribosomal protein L23 is a component of ribosomes, the organelles that catalyze protein synthesis.	-2.94	-1.44
10338708	Tmem165	Transmembrane protein 165 may function as a calcium/proton transporter involved in calcium and in lysosomal pH homeostasis. Therefore, it may play an indirect role in protein glycosylation	-1.19	-2.85
10339362	Xpo7	Exportin-7	-2.19	-2.23
10338492	TrpRS	Cytoplasmic tryptophanyl-tRNA synthetase	-2.48	-2.50

1. * shows significant expression determined by fold change expression ≧ 4.0 and ≦ -4.0
2. Reference for description of gene function was NCBI and UniProt database

Table. 2. Gene expression of L-carnitine-related genes and the OCTN family genes

Probesets	Gene symbol	Description	Fold change	
			Oxidative stress	Hypoxic stress
L-Carnitine-related gene				
10460157	Cpt1a	carnitine palmitoyltransferase 1a, liver isoform	1.13	1.02
10431564	Cpt1b	carnitine palmitoyltransferase 1b, muscle isoform	-1.00	1.04
10562989	Cpt1c	carnitine palmitoyltransferase 1c	1.08	1.04
10514933	Cpt2	carnitine palmitoyltransferase 2	1.20	1.13
10419136	Cdv3	carnitine deficiency-associated gene expressed in ventricle 3	1.15	1.06
10596185			-1.02	-1.05
10510219			-1.04	-1.18
10518344			-1.03	-1.11
10481474	Crat	carnitine acetyltransferase	-1.01	1.12
10528102	Crot	carnitine O-octanoyltransferase	-1.04	-1.18
10425987	Ppara	peroxisome proliferator activated receptor alpha	-1.01	-1.03
10589076	Slc25a20	solute carrier family 25, member 20 (mitochondrial carnitine/acylcarnitine translocase, CACT)	1.01	1.11
10402579	Slc25a29	solute carrier family 25, member 29 (mitochondrial carrier, palmitoylcarnitine transporter, CACL)	1.30	-1.06
OCTN family				
10447786	Slc22a1	solute carrier family 22 (organic anion transporter) , member 1 (OCT1)	1.05	1.13
10441774	Slc22a2	solute carrier family 22, member 2 (OCT2)	-1.08	-1.15
10447773	Slc22a3	solute carrier family 22, member 3 (OCT3)	1.00	-1.09
10385893	Slc22a4	solute carrier family 22, member 4 (OCTN1)	-1.08	-1.15
10465411	Slc22a5	solute carrier family 22, member 5 (OCTN2)	1.41	1.38
10461130	Slc22a6	solute carrier family 22, member 6 (OAT1)	1.01	1.16
10451291	Slc22a7	solute carrier family 22, member 7 (OAT2)	-1.12	1.17
10461115	Slc22a8	solute carrier family 22, member 8 (OAT3)	-1.11	1.03
10465715	Slc22a9	solute carrier family 22, member 9 (OAT7)	-1.00	-1.07
10465411	Slc22a12	solute carrier family 22, member 12 (URAT1)	-1.09	-1.15
10597612	Slc22a13	solute carrier family 22, member 13 (OAT10)	-1.00	-1.16
10362692	Slc22a16	solute carrier family 22, member 16 (OCT6)	1.03	-1.02
10465303	Slc22a20	solute carrier family 22, member 16 (OAT6)	1.05	-1.05
10385883	Slc22a21	solute carrier family 22, member 16 (OCTN3)	-1.11	-1.04
10548996	Slco1a4	solute carrier organic anion transporter family, member 1a4 (OATP1A4)	1.04	1.01
10549041	Slco1a5	solute carrier organic anion transporter family, member 1a5 (OATP1A5)	1.14	-1.04
10588263	Slco2a1	solute carrier organic anion transporter family, member 2a1 (OATP2A1)	1.10	1.21
10356918	Slco6c1	solute carrier organic anion transporter family, member 6c1 (OATP6C1)	-1.04	1.01

Table. 3. Expression of genes related to insulin, UPS, and muscle wasting

Gene symbol	Description	Number of gene under Oxidative stress		Number of gene under Hypoxic stress	
		FC ≥ 1	-1 ≥ FC	FC ≥ 1	-1 ≥ FC
Insulin expression-related gene					
MAPK	Mitogen-activated protein kinase (including map2k5, map4k1, mapk12, map3k8, map3k9) (n=5)	3	2	3	2
PIK3	Phosphatidylinositol 3-kinase (including pik3r1, pik3r2, pik3cd, pik3c3) (n=4)	0	4	0	4
FOXO3	Forkhead box 3 (n=2)	0	2	1	1
Pgc-1	PPARγ cofactor-1 (n=1)	1	0	0	1
LEP	Leptin (including lep, lepr) (n=2)	0	2	2	0
Adipo	Adiponectin (n=3)	1	2	1	2
IGF	Insulin-like growth factor including its receptors and binding proteins (n=20)	5	15	5	15
Akt	serine/threonine kinase (n=20)	14	6	11	9
	Other insulin signaling-related gene (such as sgk2, arf-1, etc.) (n=14)	8	6	3	11
UPS-related gene					
	E1 ubiquitin-activating enzyme (n=6)	3	3	1	5
	E2 ubiquitin-conjugating enzyme (n=126)	75	51	63	63
	E3 ubiquitin-protein ligase (n=83)	42	41	45	38
	Gene related to proteasome (n=125)	63	62	84	41
Muscle wasting-related gene					
Pax	Paired box protein (n=11)	10	1	6	5
PDGFRα	Platelet-derived growth factor receptor (n=2)	0	2	0	2
PPARγ	Peroxisome proliferator activated receptor gamma (n=4)	1	3	1	3
IL-6	Interleukin 6 (n=3)	3	0	3	0
Myo	Myosin (n=52)	27	25	30	22
Myh	Myosin, heavy polypeptide (n=17)	9	8	11	6
Myf	Myogenic factor (n=2)	0	2	0	2
	Genes involved in fatty acid β oxidation (n=15)	6	9	6	9
	Genes related to sarcopenia (n=16)	6	10	8	8

FC: fold change

General conclusion

In chapter I, the investigation of serum L-carnitine concentrations in healthy and randomly selected diseased cats was carried out. A positive correlation between age and serum acylcarnitine level was found in healthy cats. As in diseased cats, serum total, free and acylated L-carnitine concentrations increased in cats with DM, neoplastic and cardiac disorders significantly. Furthermore, serum L-carnitine concentrations positively correlated to haptoglobin level. These findings suggest the potential of serum L-carnitine level as a disease biomarker in cats, probably associated with insulin resistance and/or oxidative stress.

For the skeletal muscle is the largest L-carnitine reservoir in the body, and circulating L-carnitine concentration can be influenced easily, the release of L-carnitine caused by muscle damage under pathological conditions is the presumed reason for disease-associated serum L-carnitine elevation. Intramyocellular L-carnitine efflux of skeletal muscles was demonstrated in chapter II through *in vitro* studies performed with murine and feline skeletal muscle cells. Intracellular L-carnitine released significantly under oxidative and hypoxic stress, suggesting hypercarnitinemia in diseased cats was probably the consequence of L-carnitine losing from skeletal muscles. In order to further understand the route for L-carnitine release, inhibition of cellular L-carnitine transporter OCTN2 was performed. As a result, the blockage of cellular L-carnitine transporter OCTN2 accelerated L-carnitine losing, indicating L-carnitine release wasn't via OCTN2. The transportation of L-carnitine via OCTN2 was verified to be unidirectional, but the efflux pathway of L-carnitine could not be identified.

By utilization of microarray technology, gene expression of murine C_2C_{12} myotubes under oxidative and hypoxic stress was analyzed to understand the underlying transcriptional regulatory factors during cellular L-carnitine releasing in chapter III. Significant up-regulated *Mrpl52* and down-regulated *RNF220* were found in both oxidative and hypoxic stress groups, indicating that intramyocellular L-carnitine efflux was accompanied with up-regulated mitochondrial transcripts and suppression of ubiquitin proteasome pathway, the primary protein degradation system. However, the relationship of intramyocellular L-carnitine release and suppression of UPS was still unclarified.

Although supplementation with L-carnitine is broadly applied in humans, there was a frequently encountered problem that digested or injected L-carnitine would be soon eliminated instead of accumulating in its major reservoir, the skeletal muscle. Although it's difficult to rule out the possibility that hypercarnitinemia noticed in diseased cats might have resulted from excessive *de novo* biosynthesis in the liver, the current study indicates serum L-carnitine elevation to be the consequence of cellular L-carnitine releasing from skeletal muscles. The release of L-carnitine stored in skeletal muscle tissues might be a self-protecting function for the anti-wasting and anti-oxidative effects of L-carnitine, but it also indicates L-carnitine losing, probably causing the decrease of whole body L-carnitine retention.

Although transcriptional microarray analysis revealed the possible association between cellular L-carnitine releasing and suppression of UPS, specific genes directly involved in cellular L-carnitine release were not found. Further research was expected to unveil this unknown mechanism underlying intramyocellular L-carnitine efflux.

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