Gene 548 (2014) 294–298

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

# Prevention of DNA damage by L-carnitine induced by metabolites accumulated in maple syrup urine disease in human peripheral leukocytes in vitro

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ARTICLE INFO

*Article history:*
Received 3 December 2013
Received in revised form 31 March 2014
Accepted 18 July 2014
Available online 18 July 2014

*Keywords:*
Maple syrup urine disease
L-Carnitine
Oxidative stress
Antioxidant-DNA damage

ABSTRACT

Maple syrup urine disease (MSUD) is an inherited aminoacidopathy caused by a deficiency in branched-chain α-keto acid dehydrogenase complex activity that leads to the accumulation of the branched-chain amino acids (BCAAs) leucine (Leu), isoleucine, and valine and their respective α-keto-acids, α-ketoisocaproic acid (KIC), α keto-β-methylvaleric acid, and α-ketoisovaleric acid. The major clinical features presented by MSUD patients include ketoacidosis, failure to thrive, poor feeding, apnea, ataxia, seizures, coma, psychomotor delay, and mental retardation; however, the pathophysiology of this disease is poorly understood. MSUD treatment consists of a low protein diet supplemented with a mixture containing micronutrients and essential amino acids but excluding BCAAs. Studies have shown that oxidative stress may be involved in the neuropathology of MSUD, with the existence of lipid and protein oxidative damage in affected patients. In recent years, studies have demonstrated the antioxidant role of L-carnitine (L-Car), which plays a central function in cellular energy metabolism and for which MSUD patients have a deficiency. In this work, we investigated the in vitro effect of Leu and KIC in the presence or absence of L-Car on DNA damage in peripheral whole blood leukocytes using the alkaline comet assay with silver staining and visual scoring. Leu and KIC resulted in a DNA damage index that was significantly higher than that of the control group, and L-Car was able to significantly prevent this damage, mainly that due to KIC.



## 1. Introduction

Maple syrup urine disease (MSUD), or branched-chain α-keto aciduria, is a classical inborn error of amino acid metabolism caused by a mutation in the gene encoding the mitochondrial enzyme complex branched-chain α-keto acid dehydrogenase. This metabolic blockage leads to the tissue and body fluid accumulation of the branched chain amino acids (BCAAs) leucine (Leu), isoleucine, and valine and the corresponding branched chain α-keto acids (BCKAs), α-ketoisocaproic acid (KIC), α-keto-β methylvaleric acid, and α-ketoisovaleric acid in addition to the corresponding α-hydroxy acids (Chuang and Shih, 2001; Strauss et al., 2006).

Patients affected by this disease can be divided into five phenotypes ranging from the classical form with a neonatal onset to milder variants with a later onset (Chuang and Shih, 2001; Strauss and Morton, 2003). Acutely sick MSUD children or adults with metabolic decompensation suffer from muscle fatigue, vomiting, anorexia, dystonia, ataxia, stupor, and acute neurologic dysfunction manifested as decreased cognitive ability, hyperactivity, sleep disturbances, and hallucinations (Schönberger et al., 2004). In this context, damage has been shown to be associated with cerebral energy deficit (Amaral et al., 2010), neurotransmitter disturbances (Tavares et al., 2000), essential amino acid deficiency in the brain (Araújo et al., 2001), and oxidative stress. Recent studies have demonstrated that MSUD metabolites stimulate lipid peroxidation and protein oxidative damage and reduce the capacity to modulate the damage associated with increased free radical production, thereby causing cell damage and malfunction (Barschak et al., 2006, 2008; Bridi et al., 2003, 2005a,b; Fontella et al., 2002; Scaini et al., 2012; Sitta et al., 2013).

The recommended therapy for MSUD patients consists of a protein-restricted diet with a low BCAA content combined with a semi-synthetic formula of essential amino acids, vitamins, and minerals without

*Abbreviations:* ANOVA, one-way analysis of variance; BCAAs, branched-chain amino acids; BCKAs, branched chain α-keto acids; DI, damage index; L-Car, L-carnitine; Leu, leucine; KIC, α-ketoisocaproic acid; MSUD, maple syrup urine disease; SPSS, statistical package for the social sciences.

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http://dx.doi.org/10.1016/j.gene.2014.07.051
0378-1119/

L-carnitine (L-Car) (Chuang and Shih, 2001; Treacy et al., 1992). It was recently verified that MSUD patients have a deficiency of L-Car (Mescka et al., 2013), a highly polar quaternary amine that plays important metabolic functions, including transport of long-chain fatty acids across the inner mitochondrial membrane for utilization in β-oxidation participation, transesterification, and the excretion of acyl-CoA esters (Derin et al., 2004; Gulcin, 2006). In addition, L-Car has demonstrated antioxidant activity by reducing and scavenging free radical formation and by enhancing the activity of enzymes involved in the defense against oxidative damage (Derin et al., 2004; Gulcin, 2006; Muthuswamy et al., 2006). Moreover, L-Car was able to prevent oxidative damage in the cerebral cortex of young rats in an induced acute MSUD model, and L-Car supplementation increased the antioxidant status in patients with inborn errors of metabolism (Mescka et al., 2011, 2013; Ribas et al., 2010a; Sitta et al., 2011).

In the present work, we extended the investigations of the damage to biomolecules in MSUD analyzing the in vitro effect of different concentrations of Leu and KIC on DNA damage in white blood cells from normal individuals using the comet assay, as well as evaluating whether L-Car is protective under such possible damage.

## 2. Materials and methods

### 2.1. Blood sample and in vitro studies

Venous blood samples were collected into heparinized vials under sterile conditions from three healthy volunteers and used for the control group and for the tested metabolite addition. Leukocytes from each control subject were pre-treated with various concentrations of Leu (100, 250, 500, 1000, 2500, and 3000 μM) and KIC (30, 60, 150, 600, 1200, and 2000 μM) for 6 h at 37 °C. These metabolite concentrations are similar to those found in the blood of MSUD patients.

### 2.2. In vitro effect of L-carnitine on DNA damage

Leukocytes from healthy volunteers were co-incubated with 250, 500, and 3000 μM of Leu or 60, 150, and 2000 μM of KIC with an L-Car concentration curve (30, 60, 90, 120, and 150 μM) for 6 h at 37 °C. The final concentrations of L-Car in the assays were based on previous findings obtained for patients with MSUD, showing that the plasma levels of this compound can vary from 30 μM at diagnosis to almost 100 μM under supplementation (Mescka et al., 2013).

### 2.3. Single-cell gel electrophoresis (comet assay)

An alkaline comet assay was performed as described by Singh et al. (1988) and was performed in accordance with the general guidelines (Tice et al., 2000). Isolated human leukocytes were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose; the agarose was allowed to set at 4 °C for 5 min. The slides were incubated in ice-cold lysis solution to remove the cellular proteins, leaving the DNA as nucleoids. After lysis, the slides were placed on a horizontal electrophoresis unit and covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). The slides were then neutralized, washed in bi-distilled water, and stained using a silver staining protocol (Nadin et al., 2001). After drying at room temperature overnight, the gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. The cells were visually scored according to the tail length and received scores from 0 (no migration) to 4 (maximal migration) according to the tail intensity. Therefore, the damage index (DI) for the cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions by at least two different individuals.

### 2.4. Statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA), followed by a Bonferroni test when the F value was significant. A p value of less than 0.05 was considered to be significant. The values are presented as the mean ± S.D. All of the analyses were performed using the Statistical Package for the Social Sciences (SPSS) software with a PC-compatible computer.

## 3. Results

Fig. 1A and B shows the in vitro effects of Leu and KIC, respectively, on DNA damage in healthy human white blood cells. All of the tested concentrations of Leu and KIC resulted in a DNA damage index (DI) that was significantly higher than that of the control [$F(6,12) = 410.4$ and $F(6,12) = 126.4$, $(p < 0.05)$], respectively. No significant difference was found with regard to the DNA damage between the Leu concentrations of 500 to 3000 μM (Fig. 1A) and between the KIC concentrations of 150 to 2000 μM (Fig. 1B). We then analyzed the in vitro effect of L-Car (30, 60, 90, 120, and 150 μM) on the DNA damage induced by 250, 500, and 3000 μM Leu and 60, 150, and 2000 μM KIC (Figs. 2 and 3, respectively). L-Car in concentrations higher than 90 μM and 120 μM reduced the DI induced by Leu [Fig. 2A: $F(6,12) = 56.88$; Fig. 2B: $F(6,12) = 267.5$; Fig. 2C: $F(6,12) = 1174$, $(p < 0.05)$] and KIC [Fig. 3A: $F(6,12) = 149.5$; Fig. 3B: $F(6,12) = 187.5$; Fig. 3C: $F(6,12) = 280.9$, $(p < 0.05)$], respectively, at all of the tested concentrations of this amino acid and alpha keto acid. A particularly large inhibitory effect

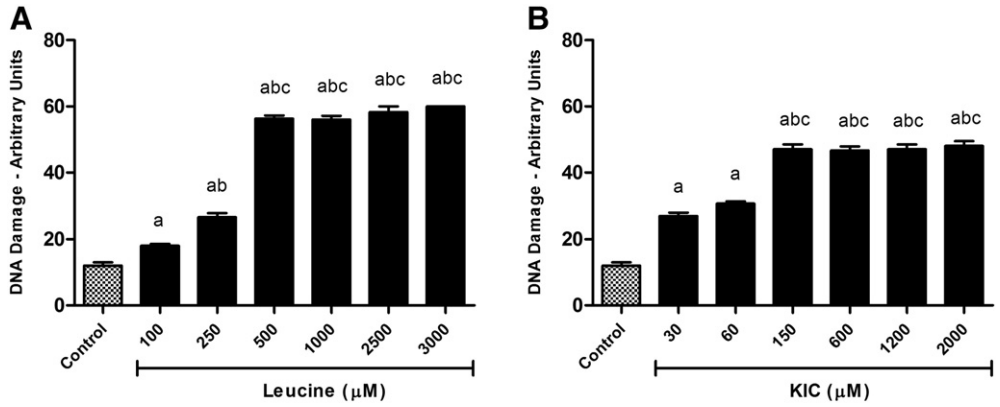


**Fig. 1.** In vitro effect of leucine (Leu) [A] and α-ketoisocaproic acid (KIC) [B] on DNA damage (comet assay) in leukocytes from whole blood. The data represent the mean ± S.D. of three independent experiments. [A]: (a) $p < 0.05$ compared to the control group; (b) $p < 0.05$ compared to 100 μM Leu; (c) $p < 0.05$ compared to 250 μM Leu. [B]: (a) $p < 0.05$ compared to the control group; (b) $p < 0.05$ compared to 30 μM KIC; (c) $p < 0.05$ compared to 60 μM KIC (one-way analysis of variance (ANOVA) test, followed by a Bonferroni test).