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Effect of chronic alcohol consumption on Hepatic SIRT1 and PGC-1α in rats

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ABSTRACT

The nuclear genes, NAD-dependent deacetylase Sirtuis 1 (SIRT1) and the peroxisome proliferator-activated receptor-γ coactivator1α (PGC-1α) are regulators of energy metabolism. Here, we studied the role of alcohol consumption in expression of these sensing molecules. Alcohol significantly reduced hepatic SIRT1 mRNA by 50% and PGC-1α mRNA by 46% and it significantly inhibited the protein expression of SIRT1 and PGC-1α, while the transcription factor PPAR-γ remained unchanged. However, when the lipid composition of the alcohol diet was changed by replacing long-chain triglycerides (LCT) with medium chain triglycerides (MCT), SIRT1 and PGC-1α mRNA were restored to near control levels. This study demonstrates that alcohol reduces key energy sensing proteins and that replacement of LCT by MCT affects the transcription of these genes. Since there is a pathophysiological link between SIRT1 and PGC-1α and mitochondrial energy, the implication of the study is that mitochondrial dysfunction due to alcohol abuse can be treated by dietary modifications.

Chronic alcohol consumption affects the liver through its metabolism resulting in an imbalance in redox equivalents, *via* alcohol dehydrogenase and generation of reactive oxygen species (ROS) *via* Cytochrome P450 2E1 (CYP2E1) [1,2].

It is well known that excessive alcohol intake generates, during its oxidation, ROS and highly reactive intermediates considered toxic for important components of the respiratory chain and mitochondrial DNA [3,4] thereby inducing mitochondrial dysfunction and liver damage [5].

However these metabolic alterations are not limited to the mitochondria since they can also activate nuclear transcription factors involved in the regulation of hepatic gene expression as in the case of fatty acid oxidation and inflammatory response [6]. Recently, it was found that a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, Sirtuis 1 (SIRT1) regulates the activity of histone [7] and non-histone [8] proteins such as the peroxisome proliferator-activated receptor-γ coactivator1α (PGC-1α), a key regulator of glucose production and energy metabolism [9]. In the liver, SIRT1 controls the gluconeogenesis/glycolytic pathway through the transcriptional coactivator PGC-1α [9]. Moreover, PGC-1α is a transcriptional coactivator that regulates several processes including energy production and utilization (thermogenesis, mitochondrial biogenesis, glucose uptake by peripheral tissues) requiring hepatocytes nuclear factor [10]. It has been shown that the nutrient control of glucose homeostasis is regulated through a complex of PGC-1α and SIRT1 [11], this interaction between PGC-1α and SIRT1 is responsible for adaptation to food deprivation and other metabolic disturbances. During prolonged fasting, the liver, which plays a critical role in the availability of fuel, activates gluconeogenesis including a rapid oxidation of fatty acids by β-oxidation, promoting generation and export of ketones bodies, an alternative source of energy. This suggests an important role of these nuclear factors in the regulation of mitochondrial biogenesis. In fact, the reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase [12].

It has been shown in cell culture that the increase in SIRT1 favors a change in the lactate/pyruvate ratio, which alters the NAD^+/NADH ratio with an increase in NAD^+. The interaction of SIRT proteins with other nuclear factors and its dependence from [NAD^+]/[NADH] ratio made it a key energy sensor responsible for transcription when there is a cellular demand according to the metabolic state of the cell [13]. Indeed it is believed that SIRT1 acts as a sensor for nutrient fluctuation *via* NAD and regulates PGC-1α-dependent gene expression [11]. This cellular imbalance is also responsible for fat accumulation associated with altered expression of PPAR-γ an important regulator factor of steatosis [14].

Recently we have shown that modification of the alcohol diet by replacement of LCT by MCT can attenuate the features of alcoholic liver damage such as CYP2E1 induction, lipid peroxidation [15] and fat accumulation suggesting that the lipid composition of the diet is an important determinant for the beneficial effect of MCT [15].

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The purpose of this study was to determine the role of chronic alcohol consumption on the expression of SIRT1, PGC-1α, and PPAR-γ in the presence of alcohol-induced liver injury. The extent to which dietary intervention can be effective in the regulation and gene expression of these proteins has also been addressed for the first time in this model of liver injury.

Materials and methods

Animals and diets. Male Sprague–Dawley rats; Charles River Laboratories (Wilmington, MA), were individually housed and fed for 28 days (in groups of six each) an equal amount of control (47% of calories as carbohydrates) and alcohol Lieber-DeCarli liquid diets, containing 36% of calories as alcohol, 11% as carbohydrates, and 18% as protein (Dyets Inc., Bethlehem, PA) [16]. A second group of rats was also fed for 21 days the control, the alcohol and an additional alcohol diet in which 32% of fat as LCT was replaced by MCT. In both experiments the food consumption was limited to the average intake of the alcohol groups fed *ad libitum* [15]. The day of the experiment, rats were anesthetized with pentobarbital (85 mg/kg intraperitoneally) for tissue collection. Animal care and experimental procedures were in accordance with animal legislation and their treatment was approved by the Animal Study Subcommittee of the James J. Peters Veterans Affairs Medical Center.

Samples preparations. Rat liver tissues from each of the treatment groups were excised, washed in saline. Part of the liver was fixed in 10% formalin for morphology, part was used to prepare liver lysate and a sterile portion (frozen immediately in liquid nitrogen) was used for mRNA extraction.

RNA isolation and real-time reverse-transcription polymerase chain reaction (RT-PCR). Liver tissue samples (20–30 mg) collected under sterile conditions and quickly frozen in liquid nitrogen, were subjected to total RNA isolation by phenol:chloroform extraction and further enriched using RNeasy mini columns (Qiagen) with digestion of RNAse-free DNAse (Qiagen).

RNA was quantified by spectrophotometer and 1 or 2 μg of total RNA was used to prepare cDNA libraries using the high Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using Taqman 2× PCR buffer (Applied Biosystems) and amplification reactions were carried out in a 7500 Thermocycler (Applied Biosystems). PCR primers for rat CYP2E1 (NM_031543), TNF-α (NM_012675), PGC-1α (NM_031347), PPAR-γ (NM_013124), SIRT1 (sequence # AT CATTCAGTGTCATGGTT) Forward prime # (GCAGGTTGCAGGAATCCAAA) Reverse prime # (GGCAAGATGCTGTTGCAAAG) constructed on demand) and 18S (X03205.1) were purchased from Applied Biosystems TaqMan. The amplification reaction was carried out in the ABI 7500 sequence detection system (Applied Biosystems) with initial hold steps (50 °C for 2 min, followed by 95 °C for 10 min) and 40 cycles of a two-step PCR (95 °C for 15 s, 60 °C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. Data were normalized relative to those for 18S RNA and changes in expression were determined using the $2^{-\Delta\Delta C_t}$ method.

Western blots and immunoprecipitation. SIRT1, PGC-1α, and PPAR-γ were measured in liver lysates. Rat liver was homogenized on ice in RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Na deoxycholate, 1% NP-40, 10 mM NaF, 5 mM Na_3VO_4, 2 mg/ml pepstatin, 2 mM PMSF, 1 mM DTT, 20 μg/ml leupeptin, and 10 μg/ml aprotinin; Upstate Cell Signaling Solution; Temecula, CA).

The homogenates were then centrifuged at 4 °C and 14,000*g* for 30 min to remove cell debris and protein aggregates and the supernatants were collected for the assay. The protein concentration was determined using the BCA protein concentration assay kit (Pierce, Rockford, IL). The primary antibodies used were: rabbit anti-SIRT1 and rabbit polyclonal anti-PGC-1α (H-3OO); sc-13067; anti-PPAR-γ (E-8); sc-7273; (Santa Cruz Biotechnology, Santa Cruz, CA). β-Actin (Sigma–Aldrich, St. Louis, MO) was used for equal loading. Immunoprecipitation of SIRT1 and PGC-1α was also carried out. Equal amounts of total protein were subjected to Immunoprecipitation by anti-Sirt1 and anti-PGC-1α conjugated to protein A agarose or protein A agarose with IgG as control.

To examine the SIRT1/PGC-1α complex, liver lysates were first immunoprecipitated with anti-SIRT1 or anti-PGC-1α and then were resolved on SDS–PAGE, transferred onto nitrocellulose membranes, and probed with the various antibodies.

Statistical analyses. Values are expressed as means ± SEM. Significant difference between mean values was evaluated using two-tailed, unpaired Student's *t* test (when two groups were analyzed) or One-way ANOVA followed by Student–Newman–Kuels post test (for 3 groups). A p value of <0.05 was considered statistically significant.

Results

Alcohol consumption and parameters of liver injury

Animals fed the alcohol diet compared to the pair-fed controls developed the alterations characteristic of chronic alcohol consumption such as fat accumulation and increase in lipid peroxidation as previously published [15]. Moreover they showed also a significant increase in the mRNA of TNF-α and Cyp2E1 ($p = 0.022$ and $p = 0.0002$, respectively) as illustrated In Fig. 1.

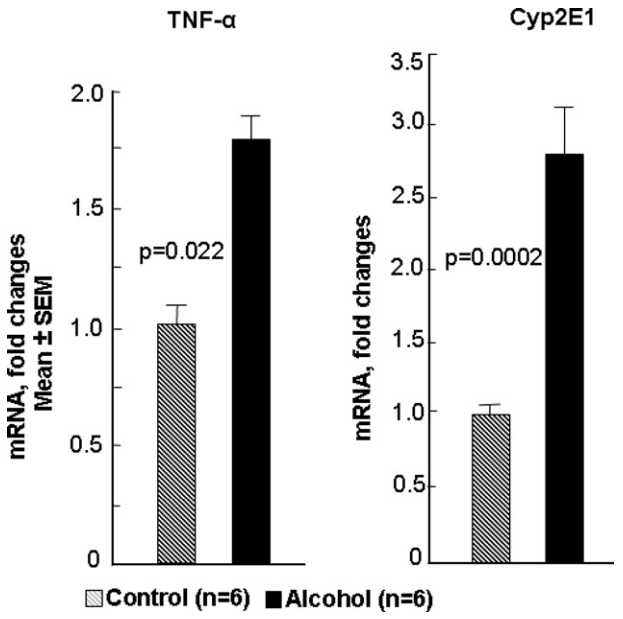


Fig. 1. Effect of alcohol consumption on parameters of liver injury. The alterations characteristic of chronic alcohol consumption such as increase in TNF-α and Cyp2E1 induction, were reproduced in this group of animals. mRNA was measured as descried in Materials and methods. Results are expressed as means ± SEM.

Alcohol consumption and key energy regulators genes: SIRT1, PGC-1α, and PPAR-γ

In the presence of the alcohol-induced liver lesions, there was a significant decrease of mRNA ($p = 0.01$) (Fig. 2A) and the relative protein levels of SIRT1 and PGC-1α ($p = 0.027$ and 0.026) without alteration of PPAR-γ (Fig. 2B). Representative Western blots of three control and alcohol animals are illustrated in Fig. 2C. Endogenous SIRT1 and PGC-1α were also immunoprecipitated with their corresponding specific antibodies or control IgG to verify the specificity of the bands (Fig. 2D). Moreover, the SIRT1/PGC-1α complex was shown by immunoprecipitation of PGC-1α protein and subsequent Western blotting for SIRT1 antibody (Fig. 2D), illustrating that these two proteins, acting as complex, can physiologically determine each others action even in this *in vivo* animal model.

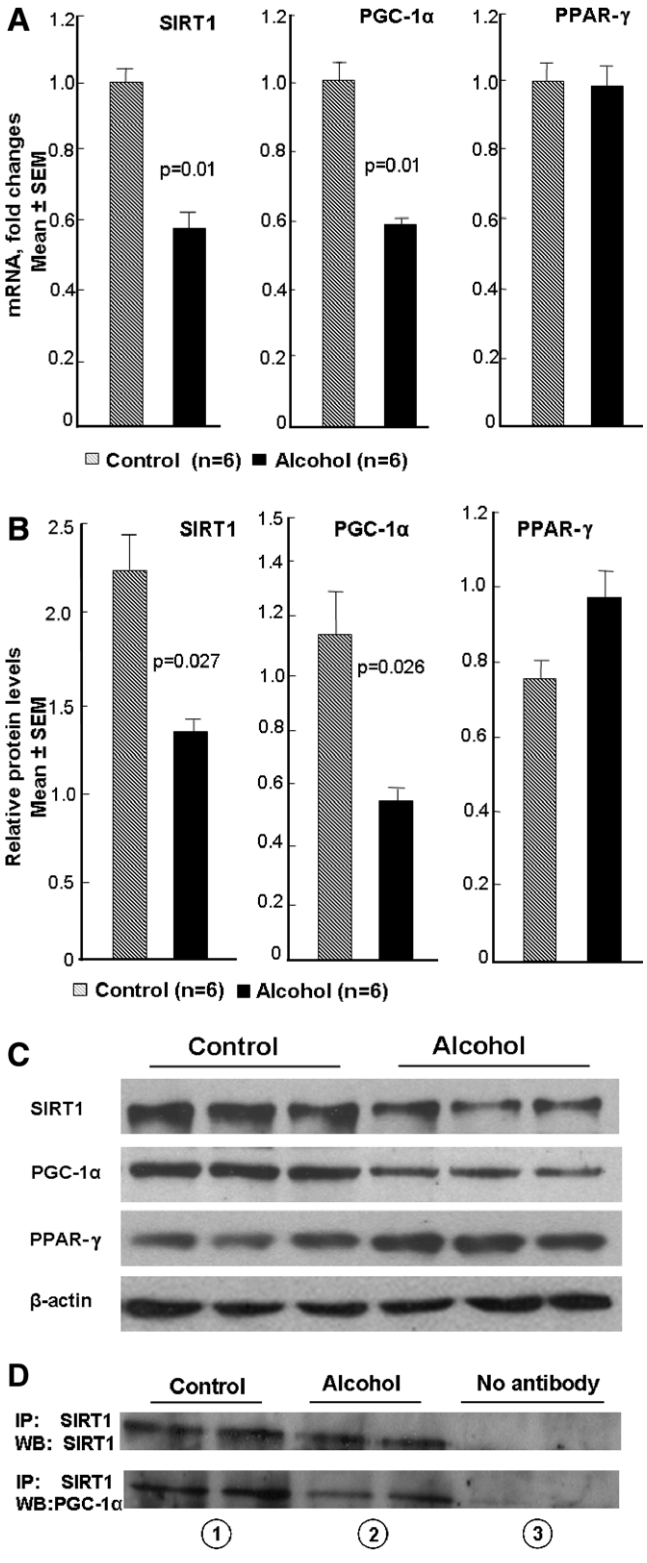
The data indicate that the alcohol-induced changes in SIRT1 protein level were regulated at the translation and post-translational level since both mRNA and protein levels were affected by chronic alcohol consumption. Since alcohol affected the levels of glucose and pyruvate, these results suggest that the decrease in SIRT1 protein could be mediated by metabolic changes due to alcohol metabolism.

The changes in SIRT1 protein level were paralleled by the decrease of PGC-1α, suggesting that these two proteins interact and are possible sensors for any NAD^+ fluctuation occurring after chronic alcohol consumption.

Effect of alcohol and MCT on hepatic SIRT1 and PGC-1α levels

In another group of rats fed the control, the alcohol and alcohol plus MCT diets, again, both mRNA and protein levels of SIRT1 and PGC-1α were significantly affected by chronic alcohol consumption and replacement of LCT by MCT prevented the alcohol-induced

depletion in mRNA, bringing it to near normal levels (Fig. 3A). However, MCT did not have any effect on the post-translation level of these proteins since it did not change their relative value (Fig. 3B and C) The beneficial role of MCT in the hepatic gene expression of these proteins demonstrate a favorable role on mitochondrial biogenesis. PPAR-γ was not significantly affected by alcohol, as shown on Fig. 2A and B, therefore was not shown for the MCT experiment.



Discussion

Our results show, in an *in vivo* model of chronic alcohol consumption, alterations at the translation and post-translational level of SIRT1 and PGC-1α, key nuclear proteins involved in energy regulation and mitochondrial biogenesis.

In fact it has been shown that, in response to fasting signals, SIRT1 induces gluconeogenic genes and hepatic glucose output, but represses glycolytic genes through deacetylation of PGC-1α [11]. The interest of these energy sensing proteins is derived from the evidence that they are regulated by several factors including food intake and are precisely up-regulated by food restriction [17].

We are proposing in this study that the decrease in SIRT1 protein is another consequence of alcohol metabolism.

Indeed, many of the toxic effects of alcohol have been linked to its metabolism. The main pathway for the oxidation of ethanol precedes *via* alcohol dehydrogenase (ADH) which results in the production of acetaldehyde and reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide-reduced form (NADH). The large amount of NADH generated overwhelms the hepatocyte's ability to maintain redox homeostasis and a number of metabolic disorders ensue, including steatosis [18]. Since SIRT1 is an NAD-dependent deacetylase, its reduction, demonstrated here, can exacerbate even further the alcohol-induced changes in the lactate/pyruvate ratio which alters the NAD^+/NADH ratio with a decrease in NAD^+ contributing in part to the alcohol-induced mitochondrial dysfunction.

In fact, chronic alcohol consumption is responsible for a food restriction and alcohol, through its metabolism, generates alteration of the redox state of the hepatocytes with reduction of NAD^+ and increase in hydroxyradical when the CYP2E1 pathway of ethanol metabolism takes over [2].

These factors are determinant of alcohol-induced reduction of SIRT1 since its deacetylase activity is dependent on NAD^+.

Moreover, the decreases in SIRT1 probably determine the reduction of PGC-1α by altering the complex function of the two proteins, acting as sensors for any metabolic change. It is well established that chronic alcohol consumption is responsible for liver damage through oxidative stress and that mitochondria are vulnerable organelles particularly susceptible to reactive oxygen species. Indeed, both nuclear and mitochondrial encoded genes are affected by chronic alcohol consumption leading to a defect in the corresponding metabolic pathway with a decrease in the level of oxidative phosphorylation in the mitochondria [19]. How the mitochondria genome responds to hypoxia is not well known; however mitochondria consume almost all O_2 available to the cells, that is why these organelles are considered as O_2 sensors [20].

The decrease of SIRT1 and PGC-1α proteins found in this study can explain the downregulation of mitochondrial DNA [21] when these organelles are sensing a change in O_2, NAD^+ or other intracellular components [22]. While PPAR-γ, proposed as a protein

Fig. 2. Effect of alcohol consumption on key energy regulator genes: SIRT1, PGC-1α, and PPAR-γ. Chronic alcohol consumption reduced mRNA (A) and protein levels (B) of SIRT1 and PGC-1α without affecting PPAR-γ. mRNA was analyzed by RT-PCR using gene-specific primers. The relative quantitation analysis was performed by calculation of the ratio of the mRNA of the gene of interest over the amount of the internal control 18S RNA. (C) The corresponding proteins were identified in liver lysates by immunoblotting with specific antibodies. The intensity of each band was quantitated by using the Image Analysis (System MCID). Values were normalized by anti-β-actin and expressed as relative protein changes. (D) Endogenous SIRT1 and PGC-1α were immunoprecipitated with their corresponding specific antibodies or control IgG to verify the specificity of the bands. The SIRT1/PGC-1α complex was evaluated by co-immunoprecipitation: immunoprecipitation of PGC-1α protein and subsequent Western blotting for SIRT1.

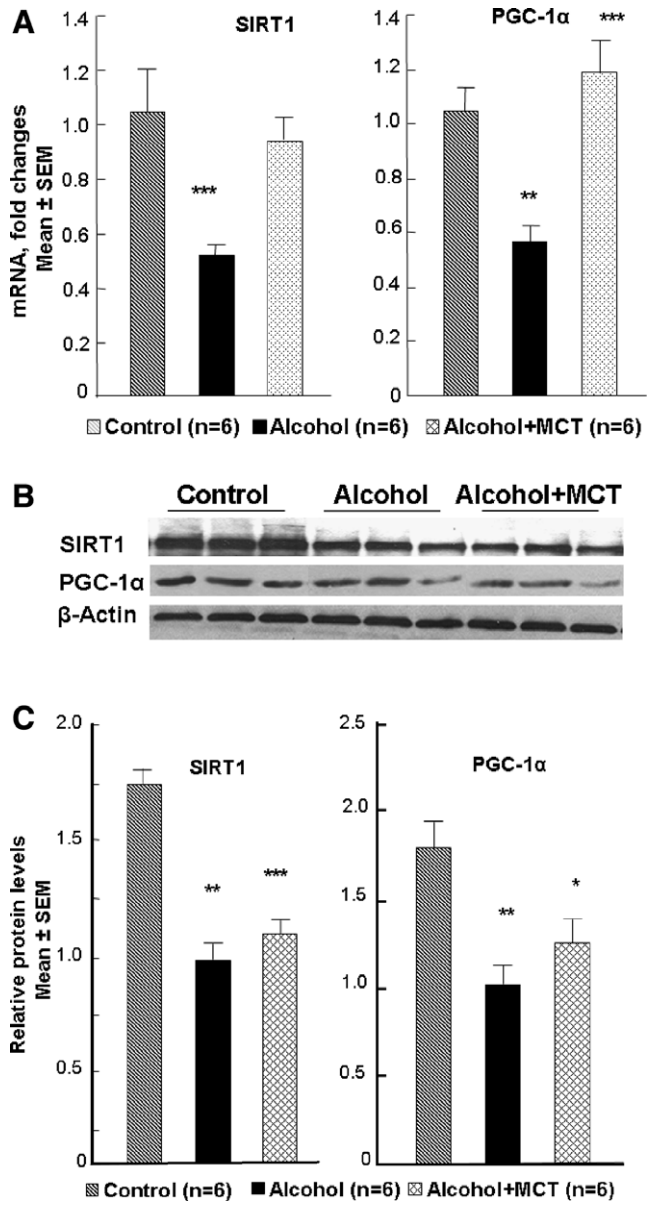


Fig. 3. Effect of alcohol and MCT on hepatic SIRT1 and PGC-1α levels. The control diet (control) contained the standard Lieber-DeCarli dextrin diet with 47% of calories as carbohydrates, 18% as protein (casein) and 32% as long-chain triglycerides (LCT) (24% corn and 8% olive oils). The alcohol diets contained 36% of calories as alcohol, 11% as carbohydrates and 18% as protein. The type of fat was either 32% of calories as LCT (alcohol) or 32% of calories as MCT (alcohol + MCT). All diets contained 3% of safflower oil. (A) mRNA for SIRT1 and PGC-1α. $^{***}p < 0.001$ alcohol vs control and alcohol + MCT; $^{**}p < 0.01$ alcohol vs control; $^{***}p < 0.001$ alcohol + MCT vs alcohol, respectively. (B) Representative immunoblots of liver lysates for each group of animals. The corresponding proteins were identified in liver lysate by immunoblotting with specific antibodies. (C) SIRT1 and PGC-1α relative protein levels. The intensity of each band was quantitated as described in methods. Values were normalized by anti-β-actin and expressed as relative protein changes. $^{*}p < 0.05$ alcohol + MCT vs control; $^{**}p < 0.01$ alcohol vs control; $^{***}p < 0.001$ alcohol + MCT vs control.

expressed in adypocytes and recently emerging as an important protein with a role in the development of hepatic steatosis [14] was not significantly affected after a short alcohol intake, other gene expression changes are likely to occur and to contribute even more to mitochondrial damage.

The nutrition effect of alcohol consumption on caloric restriction and energy metabolism is well known [23], but the role of nutrition on the transcription factors is unknown. We have recently shown that diet modification, such as replacement of LCT by MCT has beneficial effect on alcohol-induced liver injury [15]. In fact, we have shown that replacement of LCT (42% of total dietary calories) with MCT attenuates hepatic steatosis in alcohol fed rats; one reason for this difference is the propensity of MCT for oxidation rather than esterification while the converse is true for LCT [24].

Replacement of LCT with MCT has been responsible for body weight reduction through more energy expenditure and satiety [25] and for this reason it was proposed for the treatment of obesity and its prevention [26] and given *to* patients with cirrhosis [27]. Moreover, MCT inhibit free radical formation and TNF-α production in rats given enteral ethanol [28] and have a protective effect on the liver and gut after administration of endotoxin [29].

Interestingly, our results indicate the important role of the diet in the pathophysiology of disease states. Indeed, after chronic alcohol consumption, the replacement of LCT by MCT corrects the gene expression of SIRT1 and PGC-1α, confirming the importance of the nutrient control of glucose homeostasis through a complex of PGC-1α/SIRT1 postulated by [11].

Although the relative protein level remain unchanged, it is conceivable that the beneficial effect may be extended to the correction of the postulated post-translation protein modification.

In conclusion, we have demonstrated here, for the first time, *in vivo*, that for an equivalent dietary intake, chronic alcohol consumption reduces key energy sensing proteins (SIRT1 and PGC-1α) and their mRNAs expression possibly through alterations of the redox state and mitochondrial dysfunction, common features of alcoholic liver disease.

In the presence of alcohol consumption replacement of LCT by MCT affects transcription of these genes by restoring the mRNA levels to near control values. Even if MCT was not effective at the translation level since it did not increase the protein expressions we can however presume that it can correct the alcohol-induced protein modification, crucial to the proper metabolic functions.

It has been shown that because of the involvement of Sirtuis protein in multiple cellular functions, they could represent therapeutic targets to treat metabolic disorder [13]. We, then, would like to extend the mode of action of these genes and their possible association to alcohol-induced liver disease and we propose that they can be a target for nutritional therapeutic intervention.

Acknowledgments

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