ENGLISH VERSION: PATHOPHYSIOLOGIC MECHANISMS OF THE INFLUENCE OF TISSUE RESPIRATION ENZYMES ON THE MITOCHONDRIAL FUNCTION IN PATIENTS WITH CHRONIC HEPATITIS C

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Studies conducted over several decades in the field of pathophysiological mechanisms of hepatocyte mitochondria have usually been directed to functional studies of isolated mitochondria in the absence of ADP. In many cases, researchers used data to calculate parameters, including the respiratory rate or the amount of ADP consumed for each amount of oxygen used. However, so far, little is known about how the virus can survive in a highly oxidizing environment, given that oxidative stress is such an outstanding clinical feature that is associated with infection with the hepatitis C virus. In our opinion, adaptation to oxidative stress is a pathophysiological mechanism for the survival of the virus. The objective is to research mechanisms of energy supply disturbance as a mechanism of damage to cells in patients with chronic viral hepatitis C. The 62 HCV+ patients and 24 healthy controls were enrolled in the present cross-sectional study. The patients were selected on the basis of their stable clinical condition over the past 3 months. The HCV infection was diagnosed by the positivity of anti-HCV and HCV-RNA for at least 6 months of period. Mitochondrial integrity was assessed by cytochrome C release using a commercial kit (Cytochrome C Oxidase Assay Kit, Sigma-Aldrich, St. Louis) indicating a mean of 96% intact mitochondria. Intrinsic NADH fluorescence was monitored in isolated mitochondria as a marker of the mitochondrial NADH redox state. Mitochondrial division is a key determinant of mitochondrial quality control, and HCV modulates these key processes in the adaptation to cellular physiological perturbations associated with infection to promote viral persistence. Mitochondrial division is not invariably associated with cell death but can also protect cells from death induced by oxidative stress and Ca^{2+}-dependent apoptotic stimuli. The mechanism by which enzymes for energy metabolism suppress the replication of the hepatitis C virus is not yet clear, but it probably includes calcium and dissociation of the mammalian replication complex. A detailed understanding of the mechanism by which energy enzymes suppress the replication of HCV infection require additional research.

Key words: hepatitis C, NAD/NADH2, mitochondrial fission.

Introduction

The hepatitis C virus affects 3% of the world population and causes a clinically important disease [1, 2]. Hepatitis C virus is recognized as a major factor in fibrosis and cirrhosis development. Parenchymal damage of cell membranes may lead to metabolic disorders, which plays a major role of liver fibrosis in formation of hepatitis C [2-3]. The researches dating back to several decades have usually been carried out in functional studies of isolated mitochondria in the absence of ADP [1]. In many cases, investigators have used the data to calculate parameters including the respiratory control ratio or the amount of ADP consumed per amount of oxygen utilized [1]. Such studies have been widely applied to describe mitochondrial function as affected by a myriad of physiologic or pathophysiologic states.

The components of metabolomic function of hepatocytes: mitochondrial function, respiratory states and endogenic intoxication have been assessed in the past by different methods designed to regenerate ADP for phosphorylation. These include the use of creatine, creatine kinase [2–4], ATPase with excess ATP [2, 5–7], and ratio $NAD^+/NADH_2$, glucose/hexokinase [4, 8].

Most of these studies were directed at liver mitochondria. HCV establishes a chronic infection in the face of an active immune response and the host oxidative defense. However, little is known about how the virus can survive in a highly oxidative environment given that oxidative stress is such a prominent clinical feature associated with hepatitis C infection [6-12]. Adaptation to oxidative stress is key to virus survival.

The objective is to research mechanisms of energy supply disturbance as a mechanism of damage to cells in patients with chronic viral hepatitis C.

Material and Methods

62 HCV+ patients and 24 healthy controls were enrolled in the present cross-sectional study. The patients were selected on the basis of their stable clinical condition over the past 3 months.

The study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989. All subjects were informed about the study and the written consent was obtained from each one.

HCV infection was diagnosed by the positivity of anti-HCV and HCV-RNA for at least 6 months period.

Exclusion criteria

History of alcohol abuse, smoking habit, pregnancy, and antioxidant use, fish-oil or iron supplement in the previous month, receiving antiviral and/or interferon therapy, uncontrolled elevated blood pressure, serum total bilirubin level higher than 2 mg/dL, concomitant chronic hepatitis B or other well-known liver diseases such as metabolic or autoimmune disorders and various infectious states of the liver, human immune deficiency virus infection, diabetes mellitus, chronic respiratory insufficiency, rheumatoid arthritis, cirrhosis, or malignant tumor.

Virological studies

Anti-HCV was assayed by micro particle ELISA method (Anti-HCV ELISA Kit, Diagnostic Automation/Cortez Diagnostics, Inc., USA). HCV-RNA was determined using real time polymerase chain reaction (RT-PCR) method (RoboGene® HCV RNA Quantification Kit, Analytik Jena) in BioRad ICycler. Upper and lower detection limit (68 IU/ml).

Preparation of erythrocytes mitochondria

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Erythrocytes were washed twice in phosphate-buffered saline (145 mM NaCl, 5 mM NaPi, and 1 mM EDTA, pH 7.4) and white cells were removed by filtration through cellulose [9]. Total erythrocytes mitochondria were prepared by differential centrifugation and purification on Percoll [10]. Mitochondrial integrity was assessed by cytochrome C release using a commercial kit (Cytochrome C Oxidase Assay Kit, Sigma-Aldrich, USA).

The study of the ADP/ATP ratio using the ADP/ATP Ratio Assay set is a simple and direct procedure for measuring ADP and ATP levels in cells to determine apoptosis, necrosis and cell proliferation. The analysis involves two stages. In the first stage, the working reagent lyses cells to release ATP and ADP. In the presence of luciferase, ATP reacts immediately with the substrate D-Luciferin to produce light. The intensity of light is a direct indicator of intracellular ATP concentration.

Luciferase

ATP+D-Luciferin+O_2 ----------> oxyluciferin+AMP+PPi +CO_2 + light

In the second stage, ADP is converted to ATP through an enzyme reaction. This newly formed ATP then reacts with the D-luciferin as in the first step. The second light intensity measured represents the total ADP and ATP concentration in the sample.

Registration of H_2O_2 products by mitochondria. The production of H_2O_2 was estimated by ATP production as described by Yi Li [14]. Fluorescence was measured and a quantitative estimate was made [13]. In total, in a sample containing 1.5 mM cytochrome C or ferricitohrom C, 0.5 ml of isolated cells were added to HBSS (approximately 5×10^6 cells per milliliter).

Measurement of hydrogen peroxide in mitochondria. The production of hydrogen peroxide was measured fluorometrically using Amplex Red dye (Eugene, USA) in combination with horseradish. In these experiments, the incubation medium was supplemented with 1 L of ample red, 5 ml of horseradish peroxidase and 40 ml of Cu, superoxide dismutase Zn. The presence of superoxide dismutase prevents the automatic oxidation of Amplex Red, which prevents the quantitative assessment of low H_2O_2 production rates. The production of H_2O_2 in mitochondrial suspensions was recorded as an increase in the fluorescence of the dye at 585 nm, with a wavelength of excitation of 550 nm. The dye reaction was calibrated by successive addition of known amounts of hydrogen peroxide solution or continuous infusion of H_2O_2 solution at 100-1000 pmol/min. The concentration of commercial 30% solution of H_2O_2 was calculated from optical absorption of light at 240 nM); The stock solution was diluted to 100 ml with deionized water and immediately used for calibration.

Respiration and membrane potential methods by Yi Li [14] in modification.

Mitochondria (0.05 mg/ml) were incubated at 37°C in 2 ml of ionic respiratory buffer (105 mM KCl, 10 mM NaCl, 5 mM Na2HPO4, 2 mM $MgCl_2$, 10 mM HEPES pH 7.2, 1 mM EGTA, 0.2% defatted BSA) with 5 U/ml hexokinase (Worthington Biochemical), and 5 mM 2-deoxyglucose. A tetraphenylphosphonium standard curve was performed in each run by adding tetraphenylphosphonium chloride at concentrations of 0.25, 0.5, 0.75, and 1 μM prior to the addition of mitochondria to the chamber.

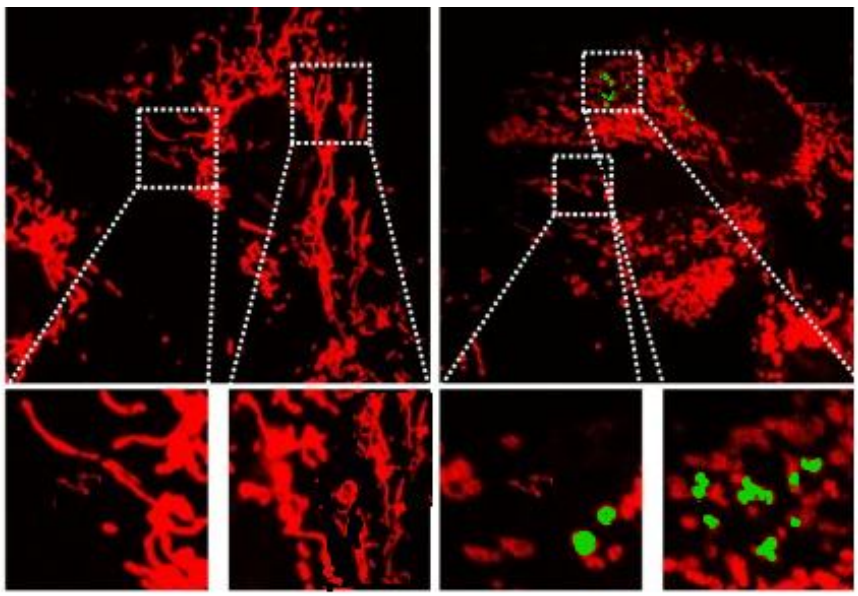
Statistical Analysis

An independent (unpaired) Student's t-test (two-tailed) was chosen to test the significance of differences among means of small “n” sample sets, using the StatGraphics Plus 3.0 package.

Results and discussion

There were no statistically significant differences between the groups with respect to age and gender ($p > 0.05$). No correlation was observed between ALT and HCV-RNA level in HCV-infection patients ($p > 0.05$).

HCV-infection induces endogenic intoxication and oxidative stress salters which causes mitochondrial dysfunction and hepatocytes damage. The HCV-infected cells displayed fragmented mitochondria distinct from non-infected cells, in which the typical tubular mitochondrial network has been observed as indicative of normal healthy cells (Figure 1).



Healthy cells **HCV-inferected cells**

Figure 1. The mitochondrial HCV-infected and healthy cells.

In the control group, we observed a gradual increase in the O_2 index when added to the ADP solution. However, when compared with a group of patients with chronic viral hepatitis C, it was found that O_2 values were significantly ($p<0.05$) higher in patients with HCV infection.

The total levels of ATP and ADP secreted the patients with hepatitis C virus was 2.25±0.35 nmoles and 3.40±0.45 nmoles. The ratio of ATP to ADP, secreted from platelets upon activation, was 1:1.60 respectively (Figure 2 (A)). Mitochondrial dynamics and quality control are closely associated with cellular metabolic alterations and ATP levels. To investigate whether inhibition in HCV secretion is a result of reduction in cellular ATP levels, we determined the total ATP levels and rate of glycolysis, an alternative mode of ATP generation..

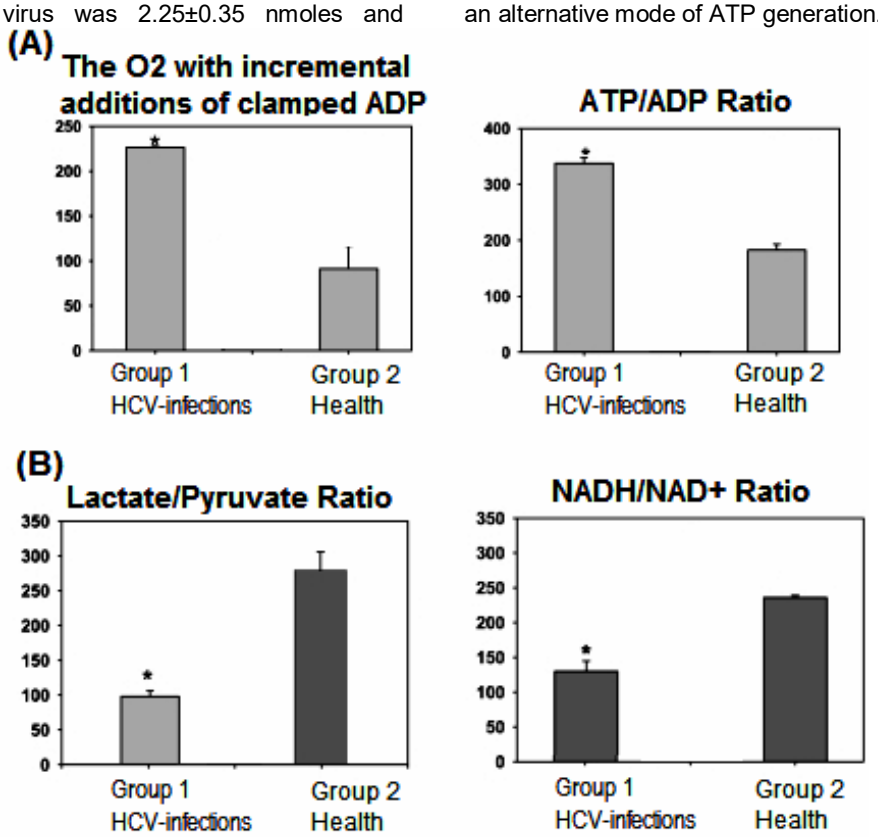


Figure 2. Inhibition of mitochondrial division and mitochondrial function. A– O2 with incremental additions of clamped ADP and ATP/ADP ratio, B – Lactate/Pyruvate ratio, NADH/NAD+ ratio..

To detect bioenergetics changes, we examined the ratio of pyruvic acid and lactate as markers of carbohydrate metabolism oxidative stage (the ratio of aerobic and anaerobic phases), and NAD^+ and $NADH_2$ levels as mandatory participants of oxidation-reduction reactions and regulators of cell metabolism. Decreased $NADH_2$ index (0.002±0.0001 mmol/l) was determined in comparison with control group (0.01+0.0005 mmol/l). The NAD^+ concentration (0.494±0.03 mmol/l) was significantly ($P<0.05$) increased in patients with HCV-infection in comparison with normal content, respectively (Figure 2 (B)). The NADH fluorescence did not change at low ADP in patients with HCV-infection. The production of reactive oxygen species (ROS) was very high in HCV-infections. An increased content of oxidized nicotinamide coenzymes was detected in patients with chronic hepatitis C.

Lactate and pyruvate parameters study has found the following. In patients of Group 1 lactate indexes exceeded the parameters of control group and amounted 2.12±0.23 and 1.89±0.45 mmol/l in comparison with control value (1.56±0.235 mmol/l). Pyruvate serum indexes were significantly lower than in the control group (0.056±0.011 mmol/l) and composed for patients with HCV-infections 0.031±0.012 mmol/l, respectively (Figure 2 (B)). Pyruvate, which re-oxidizes cytosolic NADH to NAD+, completely abrogated the increases in HCV replication.

Conclusion

Mitochondrial division is a key determinant of mitochondrial quality control, and HCV modulates these key processes in the adaptation to cellular physiological perturbations associated with infection to promote viral persistence. Mitochondrial division is not invariably associated with cell death but can also protect cells from death induced by oxidative stress and Ca^{2+}-dependent apoptotic stimuli. The mechanism by which enzymes for energy metabolism suppress the replication of the hepatitis C virus is not yet clear, but it probably includes calcium and dissociation of the mammalian replication complex. A detailed understanding of the mechanism by which energy enzymes suppress the replication of HCV infection requires additional research.

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