Hepatocyte mitochondrion electron-transport chain alterations in CCl$_4$ and alcohol induced hepatitis in rats and their correction with simvastatin

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Abstract The goal of this study was to examine the state of hepatocyte mitochondrial respiratory chain of rats with toxic hepatitis induced by CCl$_4$ and ethanol. Oxygen consumption by hepatocytes and mitochondria was determined. Endogenous oxygen consumption by pathological hepatocytes was 1.3-fold higher compared with control. Rotenone resulted in 27% suppression of respiration by pathological hepatocytes whereas 2,4-dinitrophenol produced a 1.4-fold increase of respiration. States 3 and 4 of mitochondrial respiration with malate and glutamate were found to be higher as compared to control. State dinitrophenol and state 3 respirations were similar within every group of animals when being tested with malate and glutamate or succinate. Cytochrome c oxidase activity in hepatitis was 1.8-fold higher compared with control. Simvastatin administration resulted in a decrease in hepatocyte endogenous respiration in hepatitis. The presented data lead to the assumption that the increased oxygen consumption by the respiratory chain of pathological mitochondria to be linked mainly with the altered function of complex I.

Keywords Toxic hepatitis · Hepatocytes · Mitochondrion · Respiratory chain · Lipid peroxidation · Simvastatin

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Introduction

Alcohol and toxic drugs are major causes of liver diseases in the world today (Bosch et al. 1994; Lieber 1994). Liver pathologies, particularly related to ischemic damages, are associated with significant alterations in the functional state of the mitochondrial respiratory chain (Krahenbuhl and Reichen 1992). Mitochondrial respiratory chain consists of the complex of electron transporters embedded into the inner membrane of these organelles. These electron transporters are combined in four complexes: NADH-coenzyme Q reductase (NCCR, complex I), succinate coenzyme Q reductase (SCCR, complex II), coenzyme Q-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV). ATP-synthase, located also in the inner mitochondrial membrane, uses the respiratory chain to drive ATP production (Cross 1994; Boyer 1997). Data in the literature concerning mitochondrial respiratory chain functioning in hepatitis and cirrhosis are contradictory. Some authors suppose the electron transportation by the first and second complexes to be altered in pathological hepatocytes (Krähenbühl et al. 1989; Nozu et al. 1992), whereas the others consider SCCR to be the most resistant complex which is not damaged even in liver cirrhosis (Cederbaum et al. 1974; Bottenus et al. 1982; Yang et al. 2004).

During normal intermediary metabolism and oxidative phosphorylation, mitochondria produce ROS. However, healthy cells use multiple mechanisms to limit ROS

production (Gaox et al. 2004). Progression of toxic hepatitis is associated with ROS production activation. Free radicals can cause mitochondrial respiratory chain functioning alterations (Britton and Bacon 1994). Previously, when using the model of toxic hepatitis induced by the combination of CCl_4 and alcohol, we have shown that the administration of the cholesterol-lowering drug simvastatin resulted in a decrease in the fatty liver and cholesterol concentration in the blood (Okovityy et al. 2004). Simvastatin, being an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, and involved in cholesterol biosynthesis, has an antioxidant activity (Del Puppo et al. 2001).

The purpose of this work was to study (1) the functioning of the mitochondrial respiratory chain in toxic hepatitis induced in rats by a combination of CCl_4 and alcohol and (2) the possibility of simvastatin correction of hepatocyte's mitochondria electron transport chain in toxic hepatitis rats.

Materials and methods

Materials

Rotenone and 2,4-dinitrophenol were from ICN (Aurora, Ohio). Tris-(hydroxymethyl) aminomethane was from Baker (Phillipsburg, NJ, USA). Sodium dithionite, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, hydrochloric acid and sulfuric acid were purchased from Merck (Darmstadt, Germany). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

Induction of experimental hepatitis

Male Wistar rats (140–160 g) were kept on standard rat chow and tap water ad libitum under 12-h light–dark cycles. All animals were divided into four groups. Group 1—intact, control rats; group 2—experimental hepatitis rats; group 3—experimental hepatitis rats treated additionally with simvastatin; group 4—intact rats receiving only simvastatin (simvastatin control). Control animals were kept under the same conditions without any treatment. Experimental hepatitis were induced by combination of ethanol (drinking water 5% ethanol) and intragastral delivery of 50% solution CCl_4 in vaseline oil (0.2 mg/kg body mass) twice weekly over 4 weeks via gastric cannula. Simvastatin (1 mg/kg body mass) was delivered also per os in the form of water suspension (Strubelt et al. 1978; Vengerovski et al. 2000). All animals received human care in compliance with the International Guiding Principles for Animal Research. One week after the last treatment, all animals were sacrificed under thiopental anaesthetic for analysis.

Isolation of hepatocytes

Modified Seglen's method was used to isolate hepatocytes (Seglen 1976). Briefly, when being under thiopental anaesthetic, rat liver was perfused during 5 min in situ with buffer pH 7.4 contained 8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 2 g/L glucose, 5.5 ml/L 1 M NaOH. The temperature of this solution was 37 °C. Then the liver was extracted, transferred in Petri dish and cut into approximately 1 mm^3 pieces. Pieces were washed by Eagle's medium supplemented with 0.1% glucose, 0.01 M HEPES, 1% fetal bovine serum and 40 μg/ml gentamicin and transferred into 0.5% collagenase IV solution in Eagle's medium supplemented with 0.1 M HEPES, pH 7.6. Samples were shaken with enzyme for 5 min at 37 °C. Supernatant was collected and enzyme treatment procedure of pieces was repeated a second and third time with fresh portions of collagenase. Supernatants, contained hepatocytes, were combined, and then filtered though sterile nylon filter. Hepatocytes were washed with Eagle's medium and centrifuged three times at 20 g. The viability of hepatocytes was tested with trypan blue, acridine orange, ethydium bromide and also with MMT-test (Mossman 1983). Hepatocyte viability was 70–90%.

Isolation of mitochondria

Mitochondria of the excised liver were prepared as previously described (Johnson and Lardy 1969) with slight modifications (Yang et al. 2004). The liver samples were immediately processed to obtain functional mitochondria. Liver was rinsed and fat was removed before homogenization in 30 ml of isolation buffer (250 mM sucrose without Ca^{2+}, 3 mM Tris HCL, 0.5 mM EGTA, pH 7.3). The nuclei and cell debris were removed by centrifugation at 700×g for 5 min. Then supernatants were centrifuged at 10,000×g for 10 min and the second supernatants and fat were eliminated. Pellets were resuspended in 25 ml isolation buffer without EGTA and centrifuged at 10,000×g for 10 min. This last procedure was repeated twice. The mitochondrial pellets were resuspended in 1 ml of isolation buffer without EGTA. All of the above procedures were performed at 4 °C. Mitochondrial protein was measured as described (Bradford 1976). Final mitochondrial suspensions were maintained over ice and immediately used for oxygen consumption measurement.

Assay of dry mass

The dry mass of hepatocytes was calculated by interference microscopy method (Kudriavtseva et al. 1996; Mitchison 2005) with MBIN-4 microscope (LOMO, Saint-Petersburg).

Histological protocol

Liver pieces were fixed in 4% buffered formalin, embedded in paraffin, sectioned on 5 μm thick sections, and stained with hematoxylin and eosin. The Picro-fuchsin method according to van Gieson was used to stain elastic fibers in tissue sections.

Oxygen consumption

The state of electron-transport chain of mitochondria was investigated by using polarographic determination of oxygen consumption by mitochondria and hepatocytes (Estabrook 1967; Yang et al. 2004) in the presence of specific substrates, inhibitors and uncoupling agents for different complexes of the respiratory chain. The oxygen consumption by mitochondria and hepatocytes was measured with a Clark-type oxygen electrode at 30 and 37 °C, respectively, in a 2-ml water-thermostatic incubation stirred chamber (CB1-D Hansatech). For this step, mitochondria were suspended in buffer containing 100 mM KCl, 20 mM Tris, 3 mM KH_2PO_4, 3 mM $MgCl_2$, pH 7.2 on the basis of 1 mg mitochondrial protein per 1 ml buffer. Eagle's medium was used for hepatocytes suspension to obtain a concentration of 500,000 cells per 1 ml medium.

Characteristics of mitochondrial electron-transport chain

Indices used when isolated mitochondria were analyzed

- State 4 characterizes the rate of oxygen consumption by mitochondria in the presence of 3 mM succinate (substrate of the complex II), or 5 mM glutamate and 1 mM malate (substrates of the complex I), or 5 mM ascorbate (substrates of the complex III) and 200 μM TMPD (transporter of electrons from ascorbate to cytochromes);
- State 3 characterizes the rate of oxygen consumption by mitochondria in the presence of 200 μM ADP and 3 mM succinate, or 5 mM glutamate and 1 mM malate, or 5 mM ascorbate and 200 μM TMPD.
- State dinitrophenol respiration characterizes the rate of oxygen consumption by mitochondria in the presence of 30 μM 2,4-dinitrophenol (oxidation and phosphorylation uncoupling agent) and 3 mM succinate, or 5 mM glutamate and 1 mM malate.
- Respiratory control ratio (RC)—the ratio State 3/ State 4.

Indices used when isolated hepatocytes were analyzed

- Endogenous respiration (V_0) shows the oxygen consumption by hepatocytes without exogenous substrates supplementation.
- Respiration of hepatocytes in the presence of 5 μM 2,4-dinitrophnol or 1 μM rotenone (inhibitor of the complex I).

Biochemical protocol

Cytochrome *c* oxidase activity was determined as described (Estabrook 1967). Briefly, mitochondria were suspended in incubation medium contained 100 mM KCl, 20 mM Tris, 3 mM KH_2PO_4, 3 mM $MgCl_2$, supplemented with 5 mM ascorbate (electron donor), cytochrome *c* to provide the maximal rate of the reaction and digitonin to perforated external mitochondrial membrane. Protein concentration in suspension was 1 mg per 1 ml. Cytochrome *c* oxidase activity was calculated from data concerning the rate of oxygen consumption by mitochondria when 40 μM TMPD was delivered, in accordance with the Laynuiver–Berk method (Dixon and Webb 1958).

Diene conjugates and Schiff bases were measured to study lipid peroxidation activity in liver homogenates (Bidlack and Dyel 1959; Stalnaya 1977).

Triglycerides level was determined in liver homogenates in accordance with Gottfried and Rosenberg (1973).

Statistical analysis

All calculations were made using the Statistica 6.0 program. The data obtained were presented as the mean± SE. The statistical significance of the results was determined using Student's *t*-test. A $P<0.05$ was required for the results to be considered statistically significant.

Results

Histological characteristics of the liver in experimental toxic hepatitis

Combined administration of ethanol and CCl_4 (group 2) caused moderate microvacuolar liver steatosis, necroinflammatory, proliferative activity and fibrosis. Lobular structure of liver tissue was damaged. Simvastatin treatment resulted in a decrease in steatosis, however, there was no effect on the fibrosis rate and inflammatory process in liver. Liver histological characteristics of simvastatin-treated intact animals (group 4) were identical to control.

Triglycerides determination

Development of experimental toxic hepatitis in rats was accompanied by an increase in triglycerides level in the liver to 6.5±0.8 mg per 100 mg liver (group 2) compared

with control group 1 (4.7±0.7 mg per 100 mg liver). Simvastatin application caused an authentic decrease in a triglycerides level in livers of rats of group 3 compared with group 2 (4.9±0.7 vs. 6.5±0.8 mg per 100 mg liver; $P<0.02$) and in group 4 in comparison with group 1 (2.5±0.4 mg vs. 4.7±0.7 per 100 mg liver; $P<0.05$).

Dry weight of hepatocytes

CCl_4 and ethanol administration induced a significant hepatocytes hypertrophy that caused an almost 2-fold increase in the average dry cell weight compared with control: 1,422±34 pg per hepatocyte in control (group 1) and 2,271±160 pg per hepatocyte in hepatitis (group 2; $P<0.01$). This CCl_4 and ethanol effect was significantly decreased to 1,743±170 pg per hepatocyte by simvastatin ($P<0.05$).

Condition of mitochondrial respiratory chain of isolated hepatocytes

The rates of oxygen consumption by isolated hepatocytes of all experimental groups are presented in Table 1. One can see that the endogenous respiration rate of group 2 hepatocytes is 34% higher compared with control hepatocytes (group 1). Conversely, the rate of the endogenous respiration of group 3 hepatocytes is found to be 30% lower compared with control hepatocytes and 48% lower than endogenous respiration of group 2 hepatocytes.

2,4-Dinitrophenol produced 32, 34 and 44% respiration increases in hepatocytes of groups 1, 2 and 3, respectively, as compared with endogenous respiration of hepatocytes of the comparative experimental group. Thus, 2,4-dinitrophenol

Table 1 Analysis of oxidative metabolism of hepatocytes

Indices	Control	Hepatitis	Hepatitis + simvastatin
Respiration with endogenous substrates (nmol O atom/min per mg protein) V_0	14.0±0.8	18.8±1.7[a],*	9.9±0.3[a],*/[b],**
2,4-Dinitrophenol (5 μM), (nmol O atom/min per mg protein)	18.5±0.7	25.2±5.1	14.3±1.0
Rotenone (5 μM), (nmol O atom/min per mg protein)	8.0±0.5	13.7±1.1	8.5±0.7
Cyanide, (nmol O atom/min per mg protein)	1.4±0.9	6.0±1.0[a],**	2.1±0.9[b],*

Data are presented as the means±SE of 5 animals in each group.
*$P<0.05$; **$P<0.02$ (Student's test)
[a] Compared with control group
[b] Compared with hepatitis group

Table 2 Oxidative metabolism of mitochondria isolated from liver of rats in the presence of succinate and rotenone

Indices	Control	Hepatitis	Control + simvastatin	Hepatitis + simvastatin
State 3 respiration (nmol O atom/min per mg protein)	55.4±3.1	68.2±3.4*	50.3±3.5	51.1±7.0
State 4 respiration (nmol O atom/min per mg protein)	12.7±0.7	14.0±1.3	12.3±0.6	11.7±2.0
State dinitrophenol respiration (nmol O atom/min per mg protein)	56.0±3.0	73±6.0*	44.0±6.0	47.0±1.0
Respiratory control ratio	4.4±0.1	4.9±0.2*	4.1±0.3	4.4±0.5

Data are presented as the means±SE of 5 animals in each group.
*$P<0.05$ (Student's test) compared with control group.

stimulation of respiration was similar in all groups of animals.

Delivery of rotenone (inhibitor of respiration chain complex I) in control hepatocyte suspension (group 1) resulted in a 43% decrease in the oxygen consumption rate as compared to endogenous respiration of these hepatocytes. Rotenone-induced suppression of hepatocyte respiration in groups 2 and 3 was 27% and 15%, respectively, compared with endogenous respiration values.

Condition of mitochondrial respiratory chain of isolated mitochondria

The next step of the study was concerned with examination of the isolated mitochondrial respiratory chain. This approach provides an opportunity to test mitochondrial respiratory chain functioning without influence of the cellular membrane on substrate transport into the mitochondria.

Respiration intensity of mitochondria of the second and third groups of rats examined with succinate as substrate state 4 and RC for these experimental groups did not differ from the corresponding values for the control group 1 (Table 2). We failed to find distinctions between state 3 and state dinitrophenol respiration with succinate for mitochondria of groups 2 and 3 that would demonstrate the absence of oxidation-phosphorylation dissociation distinctions in these groups. This data is similar to that presented above for hepatocytes of experimental groups 2 and 3. Also, there were no significant distinctions between variables state dinitrophenol respiration and state 3 in the first and fourth groups that would indicate the complete coupling of oxidation-phosphorylation in the respiratory chain of group 4 mitochondria (Table 2). State 3 of the 2nd experimental group mitochondria was 23% higher as compared with

control ($P<0.05$). State 3 of the third and fourth groups did not differ from the control value.

The investigation of mitochondrial respiration with malate and glutamate showed an increase in oxygen consumption of mitochondria group 2 in state 4 by 56% ($P<0.01$) compared with control (Table 3). State 4 did not differ from the control in the third and fourth groups. The respiratory control was similar to control in all groups. We failed to find any differences between state 3 and state dinitrophenol respiration with malate and glutamate in group 2, indicating an absence in the uncoupling of oxygen consumption and ATP production (Table 3). Differences between third and fourth groups in state 3 and state dinitrophenol respiration were 34% ($P<0.05$) and 27% (n.s.), respectively. State 3 of the group 2 increased 70% as compared to the control group ($P<0.001$). State 3 of the third and fourth groups did not significantly differ from the control value (Table 3).

Cytochrome c oxidase activity

Cytochrome c oxidase activity of animals with experimental hepatitis was 80% higher ($P<0.01$) compared to the control (Fig. 1). Cytochrome c oxidase activity in the third group was similar to its activity in group 2. Simvastatin administration did not result in an alteration of cytochrome c oxidase activity.

Activity of peroxidation process in rat liver

The peroxidation study in rat liver showed that diene conjugates and Schiff bases found in liver homogenates of
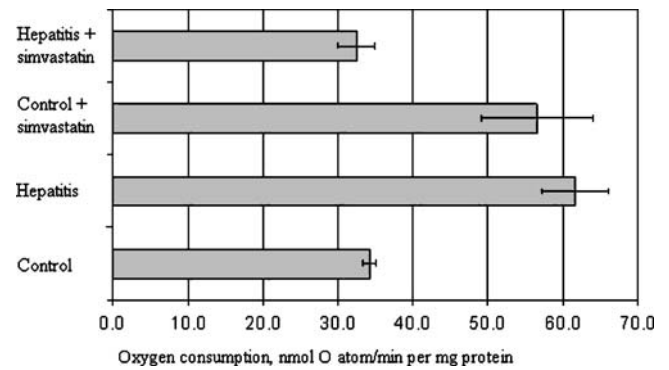

Fig. 1 Cytochrome c oxidase activity. Data are presented as the means± SE of 5 animals in each group. *$P<0.05$ vs. control (Student's test)

hepatitis group 2 were approximately 2.5-fold ($P<0.05$) higher than the control value (Table 4). Simvastatin suppressed peroxidation activation in liver initiated by toxins: there were no distinctions of peroxidation variables between the third group and control. In the control group simvastatin resulted in additional significant suppression of the peroxidation activity.

Discussion

This study showed that the functioning of mitochondrion respiratory chain complexes I and IV in hepatitis (group 2) rats differed significantly from the control. Rotenone delivery resulted in only an insignificant decrease in the respiration of isolated hepatitis hepatocytes (Table 1). Cytochrome c oxidase activity of group 2 hepatocytes was 80% higher as compared with the control group (Fig. 1). Moreover, isolated hepatitis hepatocytes mitochondria had respiratory rates state 3 and state 4 with complex I substrates (malate and glutamate) that were higher compared to mitochondria from control animals (Table 3). The increases of respiratory rates in state 3 and state 4 were also demonstrated with succinate and rotenone, although they

Table 3 Oxidative metabolism of mitochondria isolated from liver of rats in the presence of malate and glutamate

Indices	Control	Hepatitis	Control + simvastatin	Hepatitis + simvastatin
State 3 respiration (nmol O atom/min per mg protein)	27.3±2.7	48.4±2.1[a],*	27.4±4.4	33.0±3.3
State 4 respiration (nmol O atom/min per mg protein)	4.8±0.6	7.5±0.5[a],*	6.0±0.6	5.7±0.6
State dinitrophenol respiration (nmol O atom/min per mg protein)	25±2.0	44.0±6.0[a],*	20.0±1.0[b],*	22.0±3.0[b],*
Respiratory control ratio	5.7±0.5	6.5±0.1[a],*	4.6±0.5	5.8±0.2

Data are presented as the means±SE of 5 animals in each group.
*$P<0.05$ (Student's test)
[a] Compared with control group
[b] Compared with indices of the same group state 3 respiration

Table 4 Peroxidation activity in liver of rats

Indices	Control	Hepatitis	Control + simvastatin	Hepatitis + simvastatin
Shiff bases, relative units	172±22	421±49[a],**	99±7[a],*	102±15[a],*/[b],***
Diene conjugates, µmol/l	12.5±1.8	31.6±2.1[a],***	4.5±0.8[a],**	18.6±2.7[b],**

Data are presented as the means±SE of 6 animals in each group.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's test)
[a] Compared with control group
[b] Compared with hepatitis group

were significantly lower. There was a suggestion that complexes II and III are involved in this pathology. In parallel, with increased respiration rates variable state 3 and state 4, there was a 34% increase in endogenous hepatocyte respiration (Table 1). Several questions are raised in connection with this data. Why does oxygen consumption increase in the mitochondria of animals with toxic hepatitis? Is there a link in the oxygen consumption increase with the functioning of respiratory chain complexes?

Alterations in the hepatocyte mitochondrion respiratory chain complex I by pathology, in particular by cirrhosis, were described (Jikko et al. 1984; Krähenbühl et al. 1992, 2000; Yang et al. 2004). There was a suggestion that an increased level of ROS caused NCCR function alterations (Kowaltowski and Vercesi 1999; Genova et al. 2001; Kushnareva et al. 2002; Yang et al. 2004).

ROS have a high reactivity and are able to initiate free radical oxidation of cellular membrane proteins and lipids, in particular the inner mitochondrial membrane, where respiratory chain complexes are localized (Krähenbühl and Reichen 1992; Kowaltowski and Vercesi 1999). Fe^{2+}-containing mitochondrion complexes are also involved in oxidative damage of membrane lipids (Tangeras et al. 1980; Minotti and Aust 1987; Bacon and Britton 1990; Hermes-Lima et al. 1995).

CCl_4 and ethanol used for liver pathology modeling are also powerful activators of lipid peroxidation (Slater 1966; McCay et al. 1980; Chiarpotto et al. 1981). Our data on diene conjugates and Schiff bases determination are indicative of an increase of peroxidation activity by toxic hepatitis. However, there is no clarity in understanding the mechanisms of damage of mitochondrial respiratory chain complex I functioning.

Not all oxygen used by the mitochondria is involved in oxidative phosphorylation catalyzed by cytochrome *c* oxidase (Boveris and Chance 1973; Chance et al. 1979; Liu 1997; Turrens 1997). In normal conditions, 1–2% of this oxygen is used for superoxide anion production by the respiratory chain. Several sites of superoxide anion pro-

duction in the mitochondrial respiratory chain are known (Fig. 2). Admittedly, two or more such sites are in complex I (Massey et al. 1969; Fridovich 1974; Kotlyar et al. 1990; Genova et al. 2001; Kushnareva et al. 2002; Grivennikova and Vinogradov 2003; Turrens 2003). The majority of these sites were found within the flavin mononucleotide domain and domain of electron transport between Fe–S clusters and ubiquinone (Cadenas et al. 1977; Turrens and Boveris 1980). Superoxide anion production also takes place in complex III during ubiquinone oxidation–reduction (Nicholls and Ferguson 1982; Turrens et al. 1985). It is assumed that mitochondrial respiratory chain complex I is one of the key sources of ROS in the cell (Chance et al. 1979; Barja and Herrero 1998; Barja 1999; Nicholls 2002; Trojanowsk 2003). To determine the consumed oxygen portion which is not connected with cytochrome *c* oxidase functioning, we used cyanide—the specific inhibitor of this enzyme. When cyanide was delivered, the hepatocyte respiration of animals with toxic hepatitis declined to only 68% (Table 1). The oxygen consumption rate by mitochondria of toxic hepatitis group in state 3 with succinate and rotenone was not significantly higher than the control group. On the basis of these data it can be assumed complexes II and III contribute to superoxide anion production in addition to complex I. The difference of the respiration rate between hepatitis and control rats in state 3 on malate and glutamate was significantly higher as compared to the difference of the respiration rate between hepatitis and control rats in state 3 on succinate and rotenone. However, it is known that complex II has a very small capacity to produce superoxide anion as compared with the other complexes of the respiration chain (McLennan and Degli Esposti 2000). Taking into account these data we suppose complex I's contribution to superoxide anion production is significantly more in comparison with complex III. It may be assumed that the excess oxygen used in this case could be involved in a superoxide anion production increase by complex I.

Damage to this complex functioning, most likely, is associated with electron transport damage (leakage of electrons to

Fig. 2 Hypothetical scheme of oxygen consumption by mitochondrial respiratory chain of hepatitis rats
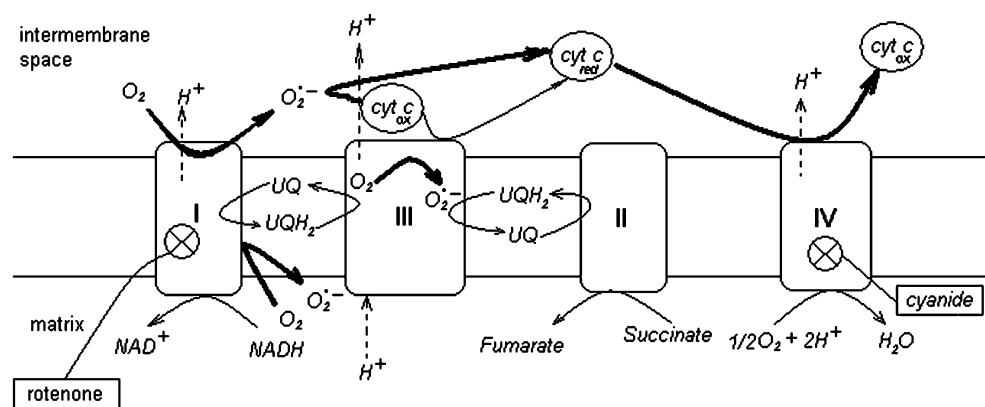
oxygen). It may cause an increase in superoxide anion production in the respiratory chain. In turn, the cytochrome c can oxidize superoxide anion passing on to the reduced state. Then, reduced cytochrome c transports the electrons to cytochrome c oxidase (Kowaltowski and Vercesi 1999).

A significant increase in cytochrome c oxidase activity in the group with toxic hepatitis, as was found in our study, is in accord with our suggestion. At the same time, oxidation and phosphorylation coupling data have shown that these reactions of coupling are normal both for substrates of complex I and for substrates of complex II. The RC was increased on both malate with glutamate and succinate with rotenone in the hepatitis group (Tables 2, 3). It seems paradoxical that toxic hepatitis mitochondria have a high RC. This fact indicates tightly coupled mitochondria. We consider a high RC to depend mainly on a high rate of respiration in state 3 that indicates a fast electron transport by the respiratory chain.

A chronic hepatitis progress scale permits an outline of some stages. One stage is the cell compensatory mechanism activation stage in response to a toxic agent. During the next stage the compensatory mechanism efforts provide hepatocytes survival for a while. At the last stage, when the cell compensatory potentialities are exhausted, the pathology progress results in irreversible alterations of structure and functions of hepatocyte components.

The analysis of scientific literature demonstrated that the usable models of toxic hepatitis have the various terms of toxic agents application. The overwhelming majority of these models have 2–6-month duration (Krähenbühl et al. 1989; Harvey et al. 2000; Yang et al. 2004) when the cellular compensatory potentialities are exhausted and the pathology process proceeds to the final stage. It indicates the importance of studying the mitochondrial respiratory chain early in the development of toxic hepatitis when the compensatory mechanism is active. Such data could help in the improvement of toxic hepatitis medical treatment.

We took into account these reasons during the choice of the duration of toxic agents application. We think that the total activation of hepatocytes compensatory mechanisms that time includes the activation of functional reserves of the mitochondria to provide increased energetic requirements of hepatocytes. Such reasons allow us to believe that increases in electron transport by the respiratory chain and in proton translocation occur to provide a higher electrochemical potential. The high rates of respiration in state 3 on malate with glutamate and on succinate with rotenone and, additionally, the high RC of mitochondria in hepatitis are in accord with our suggestion.

The high RC provides evidence for the tightly coupled mitochondria of hepatitis rats. Moreover, the tightly coupled mitochondrion is a necessary condition for ROS production by respiratory chain (Hinkle et al. 1967; Turrens 2003).

Therefore, the increase in functioning intensity of the mitochondria respiratory chain (elevated RC and cytochrome c oxidase activity) could be considered to be a compensatory mechanism, helping damaged mitochondria to cope with their ATP-producing function in conditions of high oxygen leakage for superoxide anion production.

Histological and biochemical (triglycerides, diene conjugates and Schiff bases) data found regarding simvastatin provides evidence that this medicine prevents fatty liver and decreases peroxidation in liver tissue of rats with toxic hepatitis. Additionally, simvastatin decreases the rate of endogenous respiration when using isolated hepatocytes which can be associated with its antioxidative activity and suppression of peroxidation. State 4 with malate and glutamate did not differ from the control in examined isolated mitochondria. Possibly, this is a consequence of the capability of simvastatin to inhibit peroxidation, decreasing it in such a way as to cause cellular structure damage. State 3 and state dinitrophenol respiration values demonstrated that simvastatin has a dissociative effect on the respiratory chain (Table 3). Interestingly, this effect of simvastatin became apparent only when state 3 and state dinitrophenol respiration variables were determined with complex I substrates (malate and glutamate). It may be assumed that simvastatin selectively affects complex I respiratory chain function.

The presented data permit us to hypothesize that the mechanism of toxic hepatitis promotion is connected with complex I mitochondrial respiratory chain damage (Fig. 2). This damage results in electron leakage to oxygen and superoxide anion production. Alterations in the other complex functions have a compensatory character and attempt to provide the ATP-producing function of the mitochondrion. In spite of the effect of simvastatin in the prevention of fatty liver and decreases in peroxidation in liver tissue of rats with toxic hepatitis, this drug has a dissociative effect on oxidation-reduction reactions in the respiratory chain. It can result in an overload of the mitochondrial respiratory chain. Additional experiments are needed to examine our hypothesis.

References

Bacon BR, Britton RS (1990) Hepatology 11:127–137

Barja G (1999) J Bioenerg Biomembr 31:347–366

Barja G, Herrero AJ (1998) J Bioenerg Biomembr 30:235–243

Bidlack WR, Dyel AL (1959) Lipids 8:177–182

Bosch J, Bruix J, Mas A, Navasa M, Rodes J (1994) Aliment Pharmacol Ther 8:639–657

Bottenus R, Spach P, Filus S, Cunningham C (1982) Biochem Biophys Res Commun 105:1368–1373

Boveris A, Chance B (1973) Biochem J 134:707–716

Boyer PD (1997) Annu Rev Biochem 66:717–750

Bradford MM (1976) Analytical Biochem 72:248–254

Britton RS, Bacon BR (1994) Hepatogastroenterology 41:343–348

Cadenas E, Boveris A, Ragan CI, Stoppani AOM (1977) Arch Biochem Biophys 180:248–257

Cederbaum A, Lieber C, Rubin E (1974) Arch Biochem Biophys 165:560–569

Chance B, Sies H, Boveris A (1979) Physiol Rev 59:527–605

Chiarpotto E, Olivero J, Albano E (1981) Experientia 37:396–397

Cross RL (1994) Nature 370:594–595

Del Puppo M, Galli Kienle M, Crosignani A, Petroni ML, Amati B, Zuin M, Podda M (2001) J Lipid Res 42:437–441

Dixon M, Webb EC (1958) Enzymes. Enzymes Press, New York

Estabrook RW (1967) Methods Enzymol 10:41–47

Fridovich I (1974) Adv Enzymol 41:35–97

Gaox D, Wei Ch, Chen L, Huang J, Yang Sh, Diehl AM (2004) Am J Physiol Gastrointest Liver Physiol 287:G1070–G1077

Genova ML, Ventura B, Giuliano G, Bovina C, Formiggini G, Parenti CG, Lenaz G (2001) FEBS Lett 505:364–368

Gottfried SP, Rosenberg B (1973) Clin Chem 19:1077–1078

Grivennikova VG, Vinogradov AD (2003) J Progr Biol Chem 43:19–58 (in Russian)

Harvey PJ, Gready JE, Yin Z, Couteur DG, McLean AJ (2000) J Pharmacol Exp Therap 293:641–645

Hermes-Lima M, Castilho RF, Meinicke AR, Vercesi AE (1995) Mol Cell Biochem 145:53–60

Hinkle PC, Butow RA, Racker E, Chance B (1967) J Biol Chem 242:5169–5173

Jikko A, Taki Y, Nakamura N (1984) J Surg Res 37:361–368

Johnson D, Lardy H (1969) Methods in enzymology. Academic, NY, pp 94–96 10

Kotlyar AB, Sled VD, Burbaev DS, Moroz IA, Vinogradov AD (1990) FEBS Lett 264:17–20

Kowaltowski AJ, Vercesi AE (1999) Free Radic Biol Med 26:463–471

Krähenbühl S, Reichen J (1992) Scand J Gasroenterol 27:90–96

Krähenbühl S, Stucki J, Reichen J (1989) Biochem Pharmacol 38:1583–1588

Krähenbühl S, Stucki J, Reichen J (1992) Hepatology 15:1160–1166

Krähenbühl L, Ledermann M, Lang C, Krähenbühl S (2000) Hepatology 33:216–223

Kudriavtseva MV, Emel'ianova AV, Sakuta GA, Kudriavtsev BN (1996) Tsitologiia 38:934–948 (in Russian)

Kushnareva Y, Murphy AN, Andreyev A (2002) Biochem J 368:545–553

Lieber CS (1994) Ann Med 26:325–330

Liu SS (1997) Biosc Rep 17:259–272

Massey V, Strickland S, Mayhew SG, Howell LG, Engel PC, Matthews RG, Schuman M, Sullivan PA (1969) Biochem Biophys Res Commun 36:891–897

McCay PB, Nogishi T, Lai EK (1980) Fed Proc 39:612–615

McLennan HR, Degli Esposti M (2000) J Bioenerg Biomembr 32:153–162

Minotti G, Aust SD (1987) Free Radic Biol Med 3:379–387

Mitchison JM (2005) Theor Biol Med Model 2:4

Mossman T (1983) J Immunol Methods 65:55–63

Nicholls DG (2002) Int J Biochem Cell Biol 34:1372–1381

Nicholls DG, Ferguson SJ (1982) Bioenergetics 2. Academic, London

Nozu F, Takeyama N, Tanaka T (1992) Hepatology 15:1099–1106

Okovityy SV, Yaroslavtsev MY, Sakuta GA, Litanyuk (Shiryaeva) AP, Shulenin SI, Kudryavtsev BN (2004) J Exp Hepatology 5:4–7 (in Russian)

Seglen P (1976) Meth Cell Biol 13:29–83

Slater TF (1966) Nature 209:36–40

Stalnaya ID (1977) In: Orehovich VN (ed), Modern methods in biology. Moscow, Medicine pp 63–64, (in Russian)

Strubelt O, Obermeir F, Siegers CP, Vopel M (1978) Toxicology 10 (3):261–270

Tangeras A, Flatmark T, Bäcktröm D, Ehrenberg A (1980) Biochim Biophys Acta 589:162–175

Trojanowsk JQ (2003) Exp Neurol 179:6–8

Turrens JF (1997) Biosc Rep 17:3–8

Turrens JF (2003) J Physiol 522:335–344

Turrens JF, Boveris A (1980) Biochem J 191:421–427

Turrens JF, Alexandre A, Lehningher AL (1985) Arch Biochem Biophys 237:408–414

Vengerovski AI, Baturina NO, Saratikov AS (2000) Eksp Klin Farmakol 63(2):76–80 (in Russian)

Yang S, Tan TMC, Wee A, Leow CK (2004) Cell Mol Life Sci 61:220–229