

Impairment of Mitochondrial Function of Rat Hepatocytes by High Fat Diet and Oxidative Stress

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Summary

Fatty liver disease associated with obesity is an important medical problem and the mechanisms for lipid accumulation in hepatocytes are not fully elucidated yet. Recent findings indicate that mitochondria play an important role in this process. Our data on hepatocytes in which mitochondria are in contact with other cytosolic structures important for their function, extend observations obtained on isolated mitochondria and confirm inhibition of Complex I activity in hepatocytes isolated from rats fed by high fat diet (HFD) compared with controls fed by standard diet (STD). Furthermore we have found that HFD-hepatocytes are more sensitive to the peroxidative stress because under these conditions also Complex II activity is disturbed. Therefore in HFD animals decrease of Complex I activity cannot be compensated by Complex II substrates as in STD hepatocytes. Our data thus indicates that combination of HFD and peroxidative stress potentiates HFD damaging effect of mitochondria because both branches of the respiratory chain (NADH- and flavoprotein-dependent are disturbed.

Short title: Steatotic hepatocytes are more sensitive to oxidative stress

Key words

Hepatocytes • High/fat Diet • Mitochondrial activities • ROS

Non-alcoholic fatty liver disease (NAFLD) is recognized as the most common chronic liver disease in the Western world and its primary form is considered to be the hepatic manifestation of metabolic syndrome. Although the mechanisms responsible for increased accumulation of lipids in the liver are not yet fully elucidated, decreased capacity to oxidise fatty acids, increased transport of fatty acids to the liver and increased hepatic fatty acid synthesis seem to be important factors in the pathogenesis of NAFLD (Begrache *et al.*, 2006, Wei *et al.* 2008). There are evidences that abnormalities in mitochondrial morphology and functions are involved in initiation and progression of NAFLD (Mantena *et al.* 2009, Vendemiale *et al.* 2001, Begrache *et al.* 2013). Higher formation of reactive oxygen and/or nitrogen species (ROS/RNS), decreased hepatic ATP production and induction of pro-inflammatory cytokines lead to steatohepatitis (Serviddio *et al.* 2008). Steatosis is still poorly understood since diverse effects have been reported, depending on the different model used to induce NAFLD.

Recent findings indicate that cell energy metabolism is modified by the lipotoxic effect of nutritional lipids. Vial *et al.* (2011) have shown that isolated mitochondria from rats nourished by a high-fat diet have decreased rate of mitochondrial oxidation and increased rate of mitochondrial ROS production. We decided to extend these observations and to obtain additional data, thereby supporting the hypothesis that increased ROS production participates on mitochondrial dysfunction induced by high-fat diet. We used isolated hepatocytes where mitochondria are in contact with other intracellular components that are important for their optimal functional activity (Kondrashova *et al.* 2001, 2009).

We have studied the effect of high-fat diet on the activity of mitochondrial respiratory chain function using male albino Wistar rats (Biotest, Czech Republic) housed under standard conditions as described before (Červinková *et al.* 2009). Rats of the first, control group were fed *ad libitum* by a standard pelleted diet (ST-1, Velaz, Prague, CR; 10 % energy fat, 30 % energy proteins, 60 % energy saccharides) for 6 weeks (STD). Rats of the other, experimental

group were fed by a high-fat diet (HFD; 71 % energy fat, 18 % energy proteins, 11 % energy saccharides) according to Lieber *et al.* (2004). Our previous study performed under equal experimental conditions showed that male Wistar albino rats fed by HFD developed simple steatosis without signs of progression to non-alcoholic steatohepatitis (Kučera *et al.* 2011). All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

Hepatocytes were isolated by two-step collagenase perfusion (Berry *et al.* 1991). The viability of isolated hepatocytes was more than 90 % as confirmed by trypan blue exclusion test. Respiration of isolated, digitonin-permeabilized hepatocytes was measured by High-resolution Oxygraph OROBOROS-K2 (Austria) as described before (Drahota *et al.* 2005, Červinková *et al.* 2009, Endlicher *et al.* 2009).

Digitonin-permeabilized hepatocytes were incubated at 30°C in 2 ml of a K-medium containing 80 mM KCl, 10 mM Tris HCl, 4 mM K-phosphate, 3 mM MgCl₂, 1 mM EDTA, and pH 7.4. For evaluation of oxygen uptake OROBOROS DatLab-4 software was used. For the evaluation of toxic effects of *tert*-butyl hydroperoxide (BHP) on function of mitochondrial enzyme complexes hepatocytes were incubated with 0.25 mM BHP for 5 min. before addition of respiratory substrates. 10 mM glutamate + 2.5 mM malate and 1.5 mM ADP were used for evaluation of the Complex I function and succinate was used as substrate for Complex II.

General scheme of oxygraphic measurements depicts Fig. 1. Mean values comparing STD and HFD groups with and without BHP are summarized in Table 1. After passing D'Agostino's normality test, significant differences were evaluated using unpaired Student's t-test (GraphPad Prism4.03, GraphPad Software, CA) at the selected level of significance of $p < 0.05$.

From the data presented in the Table 1 it is evident that Complex I activity was reduced in hepatocytes isolated from HFD animals, which is in agreement with the data obtained using

isolated liver mitochondria by Vial *et al.* (2011) and Mantena *et al.* (2009) . Data in Fig. 1 show that Complex I activity cannot fully saturate respiratory chain capacity because after addition of succinate as Complex II substrate, respiratory rate of hepatocytes from all experimental groups tested is further increased. In hepatocytes isolated from HFD animals succinate addition fully compensated decreased Complex I activity (Table 1). This demonstrates that Complex II activity is not affected under our experimental conditions in contrary to findings observed on isolated rat mitochondria by Vial *et al.* (2011) and isolated mice mitochondria by Mantena *et al.* (2009). This difference could be explained by lower ROS production during a shorter period of feeding animals with the high fat diet in our experiments which induces simple steatosis without inflammatory response. The other reason could be different models (mitochondria vs hepatocytes) used.

We have therefore tested whether the inhibitory effect of HFD on Complex I+II activity could be increased by combination of the high-fat diet with the peroxidative stress. In agreement with our previous findings (Drahota *et al.* 2005), we have confirmed that inhibition of Complex I by BHP in animals fed by the STD can be fully compensated by succinate (Table 1). However, the significant decrease induced by BHP and HFD was not compensated (Table 1, HFD +/- BHP). Therefore under these experimental conditions when both deleterious factors namely HFD and oxidative stress are combined also Complex II activity was depressed.

The inhibitory effect of high fat diet is quite similar to our previous observations that have shown that Complex I in comparison to Complex II, is more sensitive to the inhibitory effect of the oxidative stress (Endlicher *et al.* 2009) and to biguanides inhibitory action (Páleníčková *et al.* 2011). In these experiments, also the inhibition of Complex I was as well fully compensated by the Complex II function. However at higher concentrations of toxic agents used, inhibition of Complex II was also observed (Drahota *et al.* 2013).

We may thus conclude that changes of the cell energetics induced by the high fat diet are related to the Complex I disturbances and under normal conditions may be compensated by utilization of nutritional flavoprotein-dependent substrates, that can bypass Complex I. However when two factors, e.g. high fat diet and oxidative stress are combined the inhibition is more pronounced because under these conditions both branches of the respiratory chain (NADH- and flavoprotein-dependent) are disturbed.

Conflict of interest.

There is no conflict of interest

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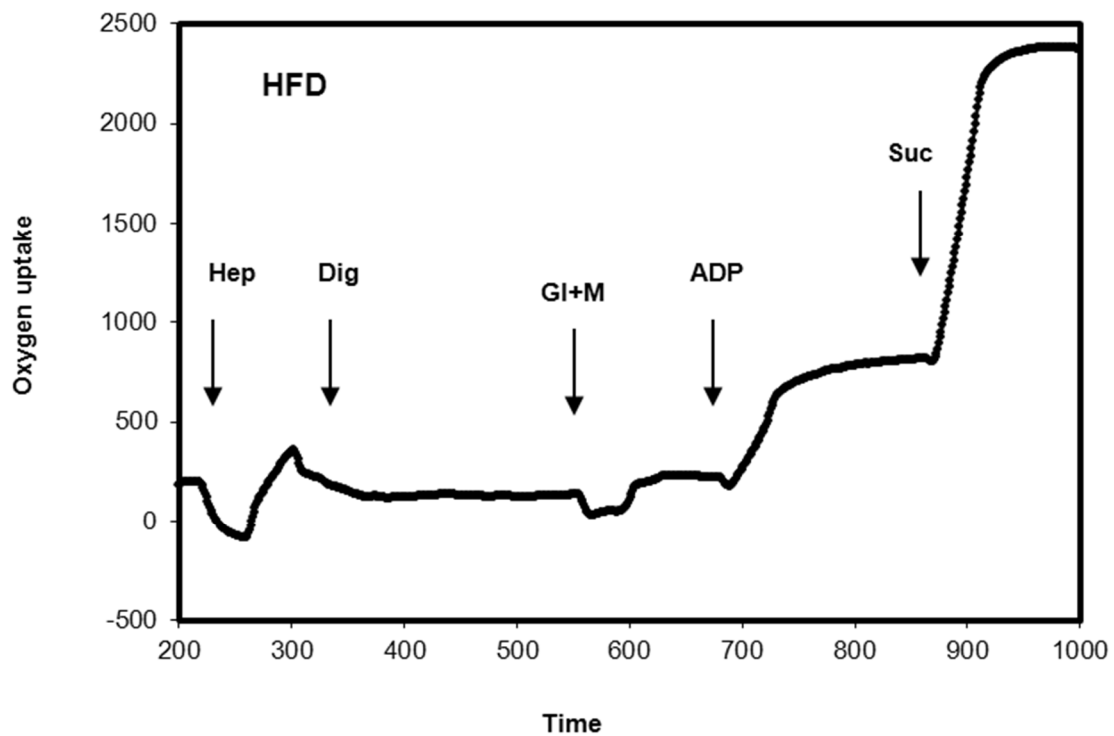
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	Gl+Mal+ADP (pmol oxygen/sec/million cells)	Gl+Mal+ADP+Suc
STD (n =15)	1073.4 ± 41.24	1764.1 ± 70.03
STD+BHP (n=11)	812.0 ± 57.14	1527.8 ± 86.44
HFD (n=12)	954.3 ± 44.98	1848.5 ± 146.63
HFD+BHP (n=10)	575.1 ± 39.84	1406.7 ± 105.72
%HFD/STD	- 11 p = 0.031	5 (n.s.)
%STD+BHP/STD	- 24 p = 0.0007	- 13 p = 0.00361
%HFD+BHP/HFD	- 40 p = 0.0199	- 34 p = 0.014

Table 1: Respiration of digitonin-permeabilized hepatocytes isolated from rats fed by standard (STD) and high-fat diet (HFD). Glutamate and malate (Gl+Mal) and ADP (Complex I activity) and after Gl+Mal, ADP and succinate (Suc; Complex I + Complex II activities) were tested under conditions described in Fig. 1. In both groups, changes of respiratory rates induced by BHP were also measured under conditions described in Fig. 1. The results are expressed as the means ± standard errors of the mean (SEM).



Text to Figure:

Fig. 1 Respiration of rat liver hepatocytes isolated from animals fed by the high-fat diet (HFD). To the 2ml of K- medium (30°C) hepatocytes (Hep) (250 000/ml) were added followed by 20µg/ml Digitonin (Dig), 10 mM Glutamate + 2.5 mM Malate (Gl+M), 1.5 mM ADP and 10 mM succinate. When BHP (0.25 mM) was tested it was added after Dig and Gl+M were added after 5 min. Oxygen uptake was expressed as pmole oxygen/sec/million cells, time in seconds.