Research Article

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Liver adapts mitochondrial function to insulin resistant and diabetic states in mice

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Background & Aims: To determine if diabetic and insulin-resistant states cause mitochondrial dysfunction in liver or if there is long term adaptation of mitochondrial function to these states, mice were (i) fed with a high-fat diet to induce obesity and T2D (HFD), (ii) had a genetic defect in insulin signaling causing whole body insulin resistance, but not full blown T2D (*IR*/*IRS-1*$^{+/-}$ mice), or (iii) were analyzed after treatment with streptozocin (STZ) to induce a T1D-like state.

Methods: Hepatic lipid levels were measured by thin layer chromatography. Mitochondrial respiratory chain (RC) levels and function were determined by Western blot, spectrophotometric, oxygen consumption and proton motive force analysis. Gene expression was analyzed by real-time PCR and microarray.

Results: HFD caused insulin resistance and hepatic lipid accumulation, but RC was largely unchanged. Livers from insulin resistant *IR*/*IRS-1*$^{+/-}$ mice had normal lipid contents and a normal RC, but mitochondria were less well coupled. Livers from severely hyperglycemic and hypoinsulinemic STZ mice had massively depleted lipid levels, but RC abundance was unchanged. However, liver mitochondria isolated from these animals showed increased abundance and activity of the RC, which was better coupled.

Conclusions: Insulin resistance, induced either by obesity or genetic manipulation and steatosis do not cause mitochondrial dysfunction in mouse liver. Also, mitochondrial dysfunction is not a prerequisite for liver steatosis. However, severe insulin deficiency and high blood glucose levels lead to an enhanced performance and better coupling of the RC. This may represent an adaptation to fuel overload and the high energy-requirement of an unsuppressed gluconeogenesis.

Introduction

The liver is intimately involved in the pathogenesis of diabetes, where hepatic insulin resistance is regarded as a key contributing element to high fasting blood glucose [1] and ketone body formation, and thus to the development of diabetic complications. Insulin resistance has been attributed to the ectopic deposition of lipids in liver and muscle [2,3], and mitochondrial dysfunction has been proposed to be the underlying cause for this lipid overload [4]. Indeed, delayed recovery from fructose-induced ATP depletion pointed to mitochondrial dysfunction in the liver of patients with steatohepatitis [5]. Moreover, low activities of respiratory chain (RC) complexes were measured in liver biopsies of patients with nonalcoholic fatty liver [6], a condition observed in about 70% of patients with obesity or type 2 diabetes [7]. Thus,

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Abbreviations: HFD, high fat diet; STZ, streptozocin; *IR*, insulin receptor; *IRS*, insulin receptor substrate; RC, respiratory chain; TCC, tricarboxylic acid cycle.

impaired energy metabolism may lead to hepatic insulin resistance and could even precede the development of hepatic steatosis in type 2 diabetes. More recently, in lean patients with type 2 diabetes, magnetic resonance spectroscopy showed lower hepatocellular ATP concentrations, which were related to low hepatic insulin sensitivity, but not to hepatocellular lipid volumes [3].

In the light of the importance of disturbed energy homeostasis and, in particular, mitochondrial dysfunction in the pathogenesis of type 2 diabetes and insulin resistance, we investigated liver mitochondrial function in three mouse models, in which glucose and lipid metabolism is affected by different mechanisms. The mice were studied at about 6 months of age or treatment, i.e., after long term insulin resistance, in order to mimic the situation encountered in the majority of patients. Even at this advanced stage, we did not observe RC dysfunction in any of our models. Therefore, no obvious relation exists between insulin signalling and mitochondrial function in the liver, in contrast to muscle, where we recently showed a severe mitochondrial impairment in muscle specific insulin receptor knockout mice (MIRKO) as well as in the same cohort of STZ mice used here [8]. However, after STZ treatment, we found an elevated capacity for substrate oxidation and a decreased mitochondrial proton leak in isolated organelles, while total hepatocellular mitochondrial content remained unchanged. We propose that this may represent an important adaptation to the fuel overload and the high energy-requirement of an unsuppressed gluconeogenesis.

Materials and methods

Animal studies

All animal studies were approved by local government authorities (Bezirksregierung Köln/LANUV). Exposure to HFD and STZ treatment, glucose and insulin tolerance tests as well as analysis of serum insulin levels, serum triglyceride and cholesterol levels were performed as described recently [8]. *IR*/*IRS-1*$^{+/-}$ mice [9] were generated by crossing mice heterozygous for insulin receptor null [10] and *IRS-1* null alleles [11] respectively, into the same C57BL/6N background. All mice were euthanized at the age of 6 months. HFD mice were fed with HFD for 5 months. STZ was injected i.p. at a dose of 60 mg/kg for five consecutive days and mice were analyzed 2 months later. In order to show hepatic insulin resistance, insulin (1.5 U/kg; Humulin R, Lilly, Indianapolis, USA) was injected i.p. in two new cohorts of HFD and *IR*/*IRS-1*$^{+/-}$ mice, the animals were euthanized by cervical dislocation 10 min later, the liver was rapidly removed and frozen in liquid nitrogen before measuring the phosphorylation state of Akt. In order to show hepatic insulin resistance in STZ mice euglycemic-hyperinsulinemic clamps were performed (see Supplementary Materials and methods).

Mitochondrial RC function

Liver mitochondria were prepared by differential centrifugation [12]. The final pellet was resuspended in polarography buffer (sucrose 250 mM, Tris 20 mM, EDTA 2 mM, KCl 40 mM, BSA 0.3%). Oxygen consumption studies were performed as described before [13]. Mitochondrial proton motive force was determined by measuring the membrane potential $\Delta\Psi$ in freshly isolated mitochondria using a methyltriphenylphosphonium TPMP ion-sensitive electrode, while analyzing in parallel the respiration rate with a Clark type electrode (Rank Bros., Cambridge, United Kingdom), in a proton leak titration assay as described in [14].

Measurement of mitochondrial enzyme activities

The carnitine palmitoyl transferase 1 (CPT-1) activity was determined as described in [12]. Since liver contains other malonyl-CoA insensitive enzymes beside CPT-2, only CPT-1 activity values are given. Spectrophotometric measurements of the TCA cycle enzymes citrate synthase (CS), isocitrate dehydrogenase (IDH), aconitase and fumarase were performed in liver homogenates using a Varian Cary 50 spectrophotometer (Varian Instruments, Mulgrave, Australia) by standard methods [15,16].

Western blots

Western blots of whole liver extracts (10 μg of protein) or liver mitochondria (2 μg of protein) were prepared. Antibodies were purchased from Cell Signaling (P-Ser^{473}AKT, total AKT), Life Technologies (RC antibodies), Biomol (MnSOD), Calbiochem (UCP-2), Sigma (β-actin), BD Biosciences (HSP-60), Santa Cruz (ANT) and used as described [8].

Lipid extraction and thin layer chromatography analysis

Lipids were extracted from frozen liver samples and analyzed as described [17].

Real time PCR

See Supplementary Materials and methods.

Microarray

Amplification and array processing (mouse Affymetrix Gene ST 1.0) were done as previously described [18]. Expression console (Affymetrix, High Wycombe, United Kingdom) was used to obtain normalized RNA gene-level data. Statistical analysis was performed in CARMAweb [19]. Genewise testing for differential expression was done employing the *t* test option and Benjamini-Hochberg multiple testing correction (FDR <10%). Pathway enrichment analysis was performed with Ingenuity Pathway software.

Other statistics

All data were compared using the two-tailed Student's *t* test assuming unequal distribution. O'Brien's OLS (ordinary least squares) test [20] was used to compare Western blot signals of complex I, III, and IV subunits.

Results

Mouse models

We have previously shown that in muscle, complete failure of insulin-transmitted signaling in STZ treated as well as in muscle-insulin-receptor knockout mice impairs RC function, while obesity-induced insulin resistance in leptin deficient *ob*/*ob* or in HFD mice has no effect [8]. Here, we have used the same cohorts of HFD and STZ-mice as well as genetically induced, insulin resistant mice (whole body *IR*/*IRS-1*$^{+/-}$ mice; animal data see Table 1) to determine whether the RC is affected in the liver. A decrease in the phospho-Akt^{473}/Akt ratio was observed in HFD-fed animals and in *IR*/*IRS-1*$^{+/-}$ mice compared to their respective controls, suggesting impaired insulin signalling in liver [9] (Supplementary Fig. 1A–B). Euglycemic hyperinsulinemic clamp studies were performed in fully conscious, freely moving, and widely undisturbed mice. STZ treated mice expressed a markedly decreased whole body insulin sensitivity compared to untreated mice (GIR, Supplementary Fig. 1E). This was accompanied by the reduced ability of insulin to suppress endogenous glucose production rates (EndoRa) from basal values in STZ treated compared to untreated individuals (Supplementary Fig. 1F). However, negative EndoRa values, frequently observed when hepatic glucose production is measured with the tracer-dilution technique during the euglycemic hyperinsulinemic glucose clamp, were calculated in control mice under