Obesity and type 2 diabetes : a systems biology perspective of a molecular mechanism

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Summary

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This thesis addressed the question whether the inhibition of the mitochondrial adenine nucleotide translocator (ANT) by intracellular long chain acyl-CoA esters (LCAC) can underlie much of the systemic phenomena observed in insulin resistant states and can link obesity to type 2 diabetes. The ANT is the mitochondrial inner membrane protein that catalyzes exchange of ADP in the cytosol for ATP in the mitochondrial matrix. Consequently, during the usual, forward mode of operation of mitochondrial oxidative phosphorylation, inhibition of this protein is expected to result in a decreased availability of cytosolic ATP, an increased level of cytosolic AMP and an increased rate of formation of extracellular adenosine. Furthermore, decreased availability of matrix ADP will result in inhibition of ATP synthesis by ATP synthase leading to rise in the mitochondrial membrane potential ($\Delta\psi$). High $\Delta\psi$ has the potential to promote the formation of reactive oxygen species (ROS). All these effects of ANT inhibition are expected to lead to impairment of cell function in ways that appear characteristic of insulin resistant states (i.e. obesity, type 2 diabetes).

Type 2 diabetes, a disease characterized by impaired glucose homeostasis is caused by defects both in insulin release from pancreatic β-cells and in insulin action on peripheral tissues. It is becoming a major health problem worldwide. Mitochondrial dysfunction is emerging as an important component of the cellular dysfunction that characterizes insulin resistant states. In many cases overweight and obesity were shown to accelerate the onset of and to exacerbate the disease. The precise role of increased adiposity in the etiology of type 2 diabetes has not yet been established. The potential roles of various adipose tissue-derived molecules such as cytokines, hormones and fatty acids are examples in terms of different individual molecular mechanisms that are investigated intensely. Due to high number of affected genes, enzymes and pathways, the traditional, i.e. reductionist, molecular biology approach to systemic diseases as obesity and type 2 diabetes may not lead to satisfactory understanding of underlying molecular mechanisms and finding potential therapeutic targets. In this thesis we take the tenet that type 2 diabetes and obesity are biological systems diseases affecting the entire energy metabolism network of the human organism. This then ends our search for a single molecular event being responsible for the etiologies of these diseases, and refocuses onto the search of effects on the pleiotropic waves of regulation through these networks. This does not remove the role of molecular actions, but it does emphasize that one should look at pleiotropic causes and effects of those. Therefore, we here focused on pleiotropic events around a single molecular action that we propose to be relevant for the link between obesity and type 2 diabetes: the inhibition of the ANT by long chain acyl-CoA esters (LCAC).

Chapter 1 of this thesis gives a general introduction to the subject. It expounds the lack of glucose homeostasis, the main characteristic of type 2 diabetes, in relation to increased adiposity. Our working hypothesis of how LCAC could bring about cellular dysfunction via inhibition of the ANT is presented. Since most of the work described in this thesis was done on isolated mitochondria, a more detailed description of parts of mitochondrial metabolism including the process of oxidative phosphorylation and the production of ROS is given. Furthermore, Metabolic Control Analysis (MCA) is introduced as a tool to quantify the control of metabolic pathway flux and metabolite concentrations by individual pathway constituents, and applied in its modular form.

In Chapters 2 and 3 the effects of two LCAC's, i.e. palmitoyl-CoA (C16:0) and oleoyl-CoA (C18:1) on oxidative phosphorylation are described. This was done in a model system consisting

of isolated rat-liver mitochondria respiring either on succinate or on the NADH-delivering substrate, i.e. combination of glutamate plus malate. To identify the enzymes of oxidative phosphorylation affected by LCAC we employed modular kinetic analysis in two different ways: (i) the system of oxidative phosphorylation was conceptually subdivided into three functional modules (substrate oxidation, phosphorylation, and proton leak) with $\Delta\psi$ as the common intermediate, and (ii) the same system was conceptually sub-divided into two functional modules (ATP-producing and ATP-consuming) with the intramitochondrial ATP/ADP ratio as the common intermediate (or the ATP/(ADP+ATP) ratio). Using this approach we found that both LCAC's inhibited the ANT and that neither had a significant effect on the other components of oxidative phosphorylation. In agreement with earlier observations, the effect of unsaturated oleoyl-CoA was weaker than that of saturated palmitoyl-CoA. Subsequently and much in the perspective of systems biology, we report that although both LCAC's acted on a single molecular target, this resulted in alterations of many steady-state mitochondrial properties. The latter included decreased oxygen uptake and phosphorylation flux, increased $\Delta\psi$, lower extramitochondrial ATP levels and higher ATP levels in the mitochondrial matrix. Quantitatively these alterations depended on the conditions under which the mitochondria were functioning.

After showing that, as hypothesized, inhibition of ANT results in lower extramitochondrial ATP availability and increased $\Delta\psi$, we then addressed the question whether this could result in increased extramitochondrial AMP formation and stimulation of ROS production (Chapter 3). We did a theoretical calculation of the extramitochondrial AMP concentration ($[AMP]_{out}$) expected at different extramitochondrial ATP/ADP ratios and showed that $[AMP]_{out}$ would increase with the decreasing extramitochondrial ATP/ADP ratios, with a stronger increase observed at low extramitochondrial ATP/ADP ratios and smaller changes at high ratios. In Chapter 2 we indeed had observed such a trend of increase in total AMP concentrations. This finding indicates that the effect of LCAC on AMP production will vary depending on the energy state of the cell. Next, we have shown that palmitoyl-CoA induces H_2O_2 production in mitochondria respiring on succinate in state 3. This process is $\Delta\psi$-dependent and largely stems from inhibition of the ANT.

The relative contribution of a particular enzyme to the control of flux through a metabolic pathway and to the control of concentrations of metabolites is one of the factors that determine the extent to which inhibition of that enzyme will affect pathway function. Accordingly, we were interested in the extent to which the ANT controls fluxes, metabolite concentrations and electric and redox potentials, and in whether these control strengths are affected by palmitoyl-CoA. In the second part of Chapter 3 we implemented MCA to quantify this control. For analysis of metabolic control we have conceptually sub-divided the system of oxidative phosphorylation into six modules (coenzyme Q-reducing module, coenzyme Q-oxidizing module, proton leak module, ATP synthesis module, adenine nucleotide translocator and hexokinase) connected by four intermediates (QH_2/Q ratio, $\Delta\psi$, matrix ATP/ADP ratio and extramitochondrial ATP/ADP ratio). This enabled us to determine the control of fluxes and intermediate concentrations not only by the ANT but also by other components of oxidative phosphorylation. Analysis of our experimental data showed that control of oxygen uptake and phosphorylation fluxes is distributed among all components of the system of oxidative phosphorylation, while the redox state of coenzyme Q (QH_2/Q ratio), $\Delta\psi$, the intramitochondrial and the extramitochondrial ATP/ADP ratios are mainly controlled by their immediate producer (positive control) and consumer (negative control). The ANT partially

controlled all investigated properties of mitochondrial oxidative phosphorylation to different extents with the largest control exerted over the intra- and extramitochondrial ATP/ADP ratios. In most instances, palmitoyl-CoA increased the absolute magnitude of the control exerted by ANT, except for the control over the QH_2/Q ratio and $\Delta\psi$, which were not significantly affected. Data presented in Chapters 2 and 3 indicate that the extent to which palmitoyl-CoA affects different mitochondrial properties can largely be explained by the magnitude of the control exerted by ANT over these properties.

Mitochondrial dysfunction is emerging as a factor in insulin resistant states. The latter often arise from an oversupply of lipids. Chapter 4 deals with the effects of long-term consumption of a high-fat diet (HFD) on mitochondrial function in rat liver as compared to the consumption of a low-fat diet (LFD). We found that 7 weeks of HFD caused accumulation of LCAC and oxidative stress as reflected by the accumulation of the marker N^{ε}-(carboxymethyl)lysine in liver. However, there were no significant changes in the mitochondrial copy number and the content of the respiratory chain components coenzyme Q, cytochromes b and $a + a_3$ (cytochrome $c + c_1$ is an exception, as a slight but significant increase was found). Furthermore, the steady-state oxygen uptake and phosphorylation fluxes, $\Delta\psi$, intra- and extramitochondrial ATP levels and the reduction level of coenzyme Q were similar in succinate-oxidizing, actively phosphorylating (state 3) mitochondria isolated from the livers of HFD- and LFD-fed rats. We used modular kinetic analysis to test whether this lack of changes in steady-state mitochondrial properties was the result of adaptive mechanisms induced in response to HFD. Such mechanisms might counteract the environmental changes by modifying the expression of involved enzymes, or by affecting the kinetics of the system in such a way that mitochondrial properties (e.g. ATP levels and $\Delta\psi$) remained unchanged. The analysis revealed that the kinetics of the modules of oxidative phosphorylation were not significantly affected by HFD treatment indicating that there was no such homeostatic adaptation at the level of oxidative phosphorylation in response to HFD treatment. Modular MCA revealed that also after HFD feeding ANT retained significant control of fluxes and intermediates of oxidative phosphorylation suggesting that LCAC should exert their acute effects on mitochondrial function also under conditions of lipid oversupply.

Endothelial dysfunction is a feature of insulin resistant states that are often characterized by high levels of circulating fatty acids. In Chapter 5 we examine the mechanisms by which fatty acids impair endothelial function and try to assess the contribution of the ANT inhibition by LCAC to these mechanisms. We showed that long term exposure of cultured human umbilical vein endothelial cells (HUVEC) to high concentrations of palmitic (C16:0) or oleic (C18:1) acid resulted in increased total LCAC content. This increase was due to accumulation of LCAC species somewhat reflecting the fatty acid type used in the incubation. The increase was accompanied by a decrease in ATP_{total}/ADP_{total} ratio. It remains to be proven that the former phenomenon is causing the second via inhibition of the ANT by the LCAC. Inhibition of acyl-CoA synthetase by triacsin C led to an increase in ATP_{total}/ADP_{total} ratio indicating that it may be the case. However, the increase in ATP_{total}/ADP_{total} ratio was observed not only in fatty acid treated but also in control cells suggesting that inhibition of acyl-CoA synthetase has a complex effect on cell function that is not easy to interpret.

Palmitic acid caused stronger activation of caspase-3 than oleic acid. In contrast to the effect of oleic acid, the effect of palmitic acid was partially blocked by antioxidant α-tocopherol

and inhibitor of ceramide synthase fumonisin B_1, and completely abolished by triacsin C. Moreover, palmitic and oleic acid differently affected cell proliferation, activation of nuclear factor-κB (NF-κB) and expression of adhesion molecules. The former process was more influenced by saturated palmitic acid, while the latter was more affected by unsaturated oleic acid. We hypothesize that this is caused by different metabolism of saturated and unsaturated fatty acids. Saturated fatty acids are preferentially converted to diacylglycerol and ceramide, molecules that regulate proliferation, differentiation and growth. However, due to toxicity of ceramide synthase inhibitor fumonisin B_1 the role of ceramides in fatty acid induced endothelial dysfunction remains to be elucidated. Unsaturated fatty acids may be more likely to contribute to oxidative stress due to oxidation of their double bonds. Taken together, we showed that depending on the type, concentration and duration of exposure fatty acids influence endothelial cell function through activation of apoptotic and inflammatory pathways.

In summary, the data presented in this thesis show that in agreement with the proposed mechanism, LCAC added to isolated rat-liver mitochondria inhibit the ANT which results in lower extramitochondrial ATP levels and higher $\Delta\psi$. This leads to increased formation of extramitochondrial AMP and ROS production by mitochondria. Furthermore, long-term exposure to a diet rich in fat causes accumulation of LCAC in rat liver indicating that the inhibition of ANT by LCAC might also occur in intact animals leading to the adverse effects demonstrated in isolated rat-liver mitochondria. This may be especially so, as long-term oversupply of lipids does not seem to lead to adaptive changes by increasing the number of mitochondria per cell or stimulating expression of ANT and/or other enzymes of oxidative phosphorylation. Similarly to rat-liver, exposure of human endothelial cells to high concentrations of fatty acids led to accumulation of LCAC and lowering of ATP_{total}/ADP_{total} ratio indicating possible inhibition of ANT. Thus our findings support the hypothesis that inhibition of ANT by LCAC can at least partly underlie cellular dysfunction characteristic of insulin resistant states (i.e. obesity and type 2 diabetes). More generally the work presented here demonstrated how modular kinetic analysis and Metabolic Control Analysis can be used in assessment of cause-effect relationships in biological systems diseases such as obesity and type 2 diabetes.