Mitochondria function and uncoupling protein-2 in rat kidney cortex; the effects of peroxisome proliferator receptor-α agonist clofibrate

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Abstract

Mitochondria are the powerhouse of the cells because of the organelles’ high energy production. The electron transport chain (ETC) consists of four complexes that are responsible for the oxidative phosphorylation. In diabetic patients there is an increase in load of substrate (NADH and FADH) to the ETC. If there is not a concomitant increase in ATP, the amount of adenosine diphosphate (ADP) will be depleted, leading to an increase of energy seeing as the proton gradient cannot be lowered. An increased mitochondria membrane potential will result in increased production of reactive oxygen species (ROS), mainly superoxide radicals. Mitochondria uncoupling (i.e. lowering membrane potential independently of ATP-production) is a known phenomenon to lower mitochondria ROS-production and is done via uncoupling protein 2 (UCP-2) in the kidney. Studies have demonstrated that peroxisome proliferator activated receptors (PPARs) can regulate UCP-2 expression, however, it is not known if this present in kidney cortex mitochondria.

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**Introduction**

Mitochondria are the powerhouse of the cells, the cellular energy is produced in this organelle. The mitochondria consists of a double membrane structure, where the outer membrane separates the mitochondria from the cytosol of the cell while the inner membrane divides the mitochondrion into two compartments, the intermembrane space and the matrix. The inner membrane of the mitochondria is a functional part in itself by housing the electron transport chain (ETC).

The ETC consists of four complexes where NADH and FADH$_2$ deliver electrons (e$^-$) to complex I and II respectively. These complexes are responsible for the oxidative phosphorylation, i.e. production of adenosine triphosphate; ATP. In the oxidative phosphorylation the electrons are delivered to complex IV where a reduction to water occurs, resulting in oxygen consumption. Electrons are charged molecules and therefore require prosthetic groups in the transferring process between complexes. Common prosthetic groups are flavin mono nucleotide (FMN), Fe-S centers, ubiquinone (CoQ) and various cytochromes. FMN is the primary e$^-$ acceptor from NADH and transfers the e$^-$ to the Fe-S centers. After the first Fe-S-center has delivered the e$^-$ to the next Fe-S and become re-oxidized, it can accept the next e$^-$ from FMNH. FMN can then accept two new e$^-$ from NADH which will start the cycle over again. Ultimately the e$^-$ is transferred through several Fe-S centers to finally reach ubiquinone, which sends the e$^-$ to complex III. From complex III cytochrome C is the responsible prosthetic group. Unlike ubiquinone which is lipid soluble and transfers e$^-$ inside the inner membrane, reduced cytochrome C is water soluble and transfers e$^-$ from complex III to complex IV via the intermembrane space. Complex IV is responsible for the reduction of molecular oxygen (O$_2$) to water according to the formula: 4e$^-+4$H$^++$O$_2$. This accounts for the oxygen consumption (QO$_2$) by mitochondria.

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**Fig 1.** The electron transport chain (ECT). Dashed arrows denotes electron donating substrates, dashed T-symbols denotes inhibitors. Modified from Friedrich et. al 2009 Diab.Curr. Rev.
Electron transfer is connected to ATP production via a proton transport in complex I, III and IV. For every two e⁻ passing through the ETC ten protons are pumped to the intermembrane space creating a chemical component due to low pH and an electrical component due to charged molecules. These two components create the mitochondria membrane potential. The ATP-synthase releases protons back across the membrane to the mitochondria matrix, releasing protons along the electrochemical gradient, releasing the energy necessary for production of ATP [1].

Diabetes mellitus affects about 4-6% of the population and is caused by reduction or absence of insulin regulation of energy metabolism and blood glucose. There are two basic forms of diabetes, insulin dependents diabetes mellitus also known as type 1 diabetes that usually occurs before the age of twenty and non-insulin dependents diabetes mellitus, type 2 diabetes that is the adult version of the disease. In type 1 diabetes the primary cause for the illness is reduction or total absence of insulin secretion due to reduction in the number of active pancreatic β-cells, in type 2 however the defect is in target cell responsiveness to insulin. Diabetes reveals itself as hyperglycemia which is natural due to the reduced insulin activity. In combination with diabetes most patients experience other complications such as hyperlipidemia that is caused by over stimulation of lipolysis and suppression of triglyceride synthesis due to lack of insulin or excess of glucagon. Lack of insulin also interferes with protein synthesis which leads to an inhibited tissue repair; this together with the lack of blood flow to the extremities is the reasons for the high infection rate in diabetic patients. Another complication that occurs more often than not is retinopathy, this is a degeneration of the retina due to lack of blood flow. The cause of diabetes is still unknown but it is thought to be related to both genetic and environmental factors, it is however known that diabetes is closely associated with oxidative stress in most tissues, especially in the kidneys [1].

An increased load of substrate (NADH and FADH) to the ETC will occur in diabetes due to intracellular hyperglycemia. If there is not a concomitant increase in ATP, the amount of adenosine diphosphate (ADP) will be depleted, meaning that an excess amount of energy is present since the proton gradient cannot be lowered by ATP production. In the case of a very high membrane potential the electrons will not be able to pass through the ETC and will connect directly to oxygen instead of being reduced to form water. This results in production of reactive oxygen species (ROS). ROS includes the free radical superoxide anion (O₂⁻), hydroxyl radical (OH⁻) and hydrogen peroxide (H₂O₂). The mitochondrion ETC always produces a basal production of ROS, mainly from complex I and III. This is normally only 0.1-0.2 % of total oxygen consumption [2-4]. However in diabetes, when the membrane potential is dangerously high the production of ROS is markedly increased [5]. A certain amount of ROS might be essential for cell survival, but the current hypothesis regarding ROS production during different pathological conditions such as diabetes is that an increase in ROS production damages the mitochondria resulting in ROS production and increased oxidative stress thereby creating a vicious circle [1].
To prevent this, mitochondria has developed a system to lower the proton gradient and prevent excessive production of ROS. This phenomenon is known as mitochondria uncoupling and is mediated by uncoupling proteins (UCP). There are five different isoforms of UCP. UCP-1 is located in thermogenic brown adipose tissue [6] whereas UCP-3 can be found in skeleton muscle [7]. UCP-2 is expressed ubiquitously throughout the body but it is also present in rats, mice and human kidneys [8-10]. Furthermore, UCP-2 protein expression in the kidney is elevated in diabetic rats [8] and may therefore play a role in the development of diabetic nephropathy.

UCP-2 facilitates a proton leak across the membrane by using charged free fatty acid molecules that bind to the protons and flip them across the membrane. When on the other side the fatty acid releases the proton and returns to the other side of the membrane trough the UCP-2 channel, the phenomenon is known as fatty acid cycling. The purpose is to modulate the mitochondrial function by lowering the mitochondria membrane potential. Mitochondria uncoupling protects the cell against oxidative stress by reducing mitochondrial generation of ROS. Indeed, UCP-2 is activated in diabetic kidneys resulting in a mitochondria uncoupling evident as increased oxygen consumption independent of ATP-production [8]. UCP-2 definitely play a role in ROS-production from mitochondria. This was nicely demonstrated by Duval et al. by utilizing small interference RNA (siRNA) towards UCP-2 showed that decreased levels of UCP-2 increased the ROS production from mitochondria [11]. UCP-2 may be an important therapeutic target as mice over expressing UCP-2 in the brain display improved recovery and brain function after brain trauma compared to wild-type mice [12].

UCP-2 in mitochondria is regulated by gene transcription, mRNA translation and activation of the protein, activation of the protein is necessary to catalyze a proton leak i.e. uncoupling. UCP-2 mediated uncoupling is stimulated by superoxide and free fatty acids, and it is inhibited by purine nucleotides such as guanosine triphosphate (GTP), ATP, guanosine diphosphate (GDP) and ADP [13]. Gene expression is regulated by different stimuli and by factors that influence the promoter. Even though the mechanism of UCP-2 regulation are not completely clear, recent research has indicated peroxisome proliferator activated receptors (PPARs) could have part in its regulation [14].

PPARs are present in three isoforms PPAR-α, PPAR-γ, and PPAR-δ. PPARs are lipid activated transcription factors that regulate the expression of target genes that are involved in energy and lipid metabolism. Where some of the isoforms (PPAR-α and PPAR-γ) have been widely studied as therapeutic targets for dyslipidemia and type 2 diabetes, PPAR-δ still remains unstudied even though it has been shown that is has ubiquitous expression [15]. At first the physiological ligands of the PPARs were unknown, but eventually their function was studied by using synthetic ligands. PPAR-α, the first isofom discovered is the isoform expressed in a variety of tissue such as adipose tissue, kidney, liver and brain. Studies have shown that there is a connection between ligand mediated activation of PPAR and activation
of the UCP-2 promoter to increase transcription [14]. The ligands used most often for PPAR-α in experiments were fibrates such as clofibrac acid and bezafibrate. The studies regarding fibrate binding to PPAR-α demonstrated a hypotriglyceridemic effect by inducing transcription of several genes that are related to oxidative metabolism of lipids [16].

Clofibrate is a PPAR-α agonist and commonly used as a hypolipidemic drug. It is known to produce peroxisome proliferation and therefore a member of the large class of peroxisome proliferators (PPs) [17]. In experiments clofibrate treatment has strongly reduced glucokinase and pyruvate kinase, and clofibrate also increases body temperature by proving it is thyromimetic [18].

It has been demonstrated that a third of all diabetic patients have diabetic nephropathy, the reason for this is still unknown. However it is known that the QO₂ as well as the uncoupling of the mitochondria increase in the diabetic kidney [8]. Research has also shown that UCP-2 is regulated by PPAR-α in liver and β-cells, but it is unknown if this regulation exist in the kidney. Interestingly animals that lack PPAR-α suffer from greater kidney damages when subjected to diabetes. The aim of the present project is to investigate whether UCP-2 is regulated via PPAR-α. This will help clarify the mechanisms underlying the development of diabetic nephropathy.

**Materials and methods**

All animal experiments and treatments were approved by the local animal care and use committee. All chemicals were from Sigma-Aldrich, Germany unless otherwise stated.

Sixteen male Sprague-Dawley rats were used, divided into two groups; eight control rats and eight treated rats. All the rats were housed in the animal facility in a light and temperature controlled environment with food and water *ad libitum*. The treated rats were given 240mg/kg clofibrate intraperitoneally once daily. It was originally planned that the rats would receive the treatment through the drinking water for two weeks; however after the first day it was clear that the substance did not dissolve properly in the water, which could mean that the intake of the substance would not be as expected, leading to incorrect results. The method was therefore changed, to intraperitoneal treatment with the clofibrate dissolved in 1ml olive oil. The treatment was repeated every day for 14-20 days at approximately the same time. The difference in days was due to the fact that all rats were not taken to experiments on the same day, all rats were treated until the day of the experiment.

Rats were killed by decapitation as commonly used anesthesia such as Inactin is detrimental for the mitochondria function. The kidneys were removed and placed on ice in 10 ml buffer A (in mmol/l: 250 sucrose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 300 mOsm/kg H₂O). The kidney capsule was removed and the cortex was
isolated, all procedures were done on ice tissue to ensure mitochondria viability. The liver was taken out, weighed and thereafter discarded. The cortex homogenized in 10 ml ice-cold buffer A with 8 complete strokes with a prechilled Potter-Elvehjem homogenizer (600 rpm). The homogenate was allocated to ten eppendorf tubes with 1.5ml in each tube and centrifuged at 800 G (4°, 10 min), the supernatant was transferred to ten new tubes and centrifuged again (8000 G, 4°, 10 min). The final supernatant was discarded and the pellet carefully washed once with buffer A and all remaining medium was removed. The pellets were then dissolved in 200 μl buffer A and stored on ice.

An oxygraph with a Clark-type electron measuring oxygen consumption at pmoles/s/ml (Oroboros Instruments,O2K, Innsbruck Austria) as calibrated with air-equilibrated buffer B (in mmol/l: 70 sucrose, 220 mannitol, 5 MgCl2, 5 KPO4−, 10 HEPES, pH 7,4, 300 mOsm/kg H2O) and used for measuring mitochondria oxygen consumption (QO2). The chambers were filled with a 2.5 ml 37°C buffer B and allowed to stabilize.

**Oxygen consumption measurements**

The mitochondria from each rat were all used in three separate experiments:

1. 20 μl of mitochondria were added to 2.5ml buffer B in the chambers and after stabilization the QO2 was denoted as “baseline”. Glutamate (10 mM) and sodium palmitate (48 μM) were added and after stabilization denoted as “glutamate”. ADP was added, when a satisfactory rise in QO2 was indicated the run was ended and a sample from the chamber was taken for later protein concentration determination.

2. 20 μl mitochondria and oligomycin (5000 μM) were added to the chambers and thereafter the same procedure as in 1. was followed. However no rise in QO2 was expected in this experiment.

3. 100 μl mitochondria was preincubated on ice with 5000 μM oligomycin and 0.5 mM GDP for 30 min. Concentration of GDP and oligomycin in the chamber was corresponding to the concentration used in the preincubation. Thereafter the experiment was conducted as experiment 1.

Control and treated animals were treated alike through all experiments, and all QO2-values were later corrected for protein content. Between every run the chambers were rinsed with
distilled H₂O four times before 2.5 ml of buffer B at the end of each day the chambers were filled with 100% ethanol and allowed to stir overnight to remove residual amount of oligomycin and GDP.

**Western blot analysis**

An approximately 0.1g kidney cortex piece intended for western blot analysis was placed in fospreptubes containing 1 ml RIPA buffer (3 ml RIPA, 1 complete miniprotease tablet (Roche Diagnostics, Mannheim Germany) and 30 µl phosphatase inhibitor cocktail II). The samples were homogenized and centrifuged on 15000 G (4°C, 10 min); the supernatant transferred to new tubes and frozen until western blot analysis.

The samples were homogenized in 700 µl buffer (1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/l NaF, 80 mmol/l Tris, pH 7.5) which contained inhibitors (Phosphatase inhibitor cocktail-2; 10 µl/ml and Complete Mini; 1 tablet/1.5 ml Roche Diagnostic, Mannheim, Germany). The samples were run on a 12.5% Tris-HCl gel with Tris/glysin SDS buffer (Bio-Rad western blot system). The proteins were detected after transfer to nitrocellulose membranes, using goat anti-rat UCP-2 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated secondary antibody (rabbit anti-goat, 1:10000; Kirkegaard and Perry Laboratories, Gaitherburg, MD). The detection was done by ECL-camera (Kodak Image station 2000; New Haven, CT). β-actin was detected by using mouse anti-rat β-actin antibody (1:10 000) (Sigma-Aldrich, Germany) and secondary HRP-conjugated goat-anti mouse antibody (1:60 000 Kirkegaard and Perry Laboratories Gaitherburg, MD). The UCP-2 protein expression was normalized for β-actin content.

**Protein analysis**

Protein concentration of western blot samples and chamber samples was determined by the DC Bio-Rad assay using absorbance of 540 nm.
Results

Mitochondria QO$_2$ did were not different between controls and clofibrate-treated animals neither during baseline nor after addition of glutamate (Fig1).

![Figure 1. Mitochondria consumption (QO$_2$) under baseline and after addition of glutamate (10 mM). Values are displayed as mean ±SEM.](image)

When depicted as glutamate-stimulated QO$_2$ both groups increased, however with no difference between the groups (Fig 2). No effect of GDP was observed (Fig 3) and UCP-2 protein levels were not significantly different between the groups (Fig 4).
**Figure 2.** Glutamate stimulation in kidney cortex mitochondria. Values are displayed as mean±SEM.

**Figure 3.** Effect of guanosine diphosphate (GDP) in kidney cortex mitochondria. Values are displayed as mean±SEM.
There was no difference in the liver weights (Fig 5), which demonstrates that there was no uptake of clofibrate.

**Figure 4.** Protein expression of UCP-2 corrected for β-actin. Values are displayed as mean±SEM.

**Figure 5.** Liver weights from control and clofibrate-treated rats. Values are displayed as mean±SEM.
Discussion

There was no effect of the clofibrate treatment in regard to UCP-2 protein expression, nor was mitochondria oxygen consumption affected. Research has demonstrated that there is a clear connection between PPAR-α and UCP-2 [19, 20], however this could not be proven in the present study. The reason for the lack of effect is likely to be in the treatment method. Earlier studies have been done where the treatment was given through the drinking water [21], however the clofibrate did not dissolve properly in the water which meant that the animals did not intake the proper amount of. Another method commonly used is intraperitoneal injection [22]. In the present study, this method proved dissatisfactorily seeing as the clofibrate precipitated in the abdomen. Thus, it is unclear if clofibrate was taken up by the bloodstream, but judging from the results it is highly unlikely. An increase in liver weight in PPAR-α treated animals is common, yet the results from the present study show no signs of this which further indicates that there was no uptake of clofibrate. GDP is a UCP-2 inhibitor. However, in the present project GDP did not display any inhibitory effect, indicating that UCP-2 is not active in these animals and UCP-2 protein content was not altered with clofibrate treatment.

Conclusion

No clear conclusion could be drawn from the present study due to the fact that the method used was not optimal. Further studies are necessary with a treatment method that ensures substance uptake.
References


