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The role of uncoupling protein-2 in regulating mitochondrial oxygen consumption in the diabetic kidney.

A regulatory mechanism by glutamine.

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Bachelor thesis in Biomedicine, 15 HP

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Abstract

Diabetes is one of our times most common diseases, affecting millions every year and it is said to stand for almost 50% of inpatient care in the United States. Diabetes has two general forms, type I, insulin dependent diabetes and type II, noninsulin dependent diabetes. Diabetes is associated with increased production of reactive oxygen species (ROS) which cause a dysfunction of the cells, a phenomenon known as oxidative stress. In the kidney increased production of ROS can activate the mitochondrial protein uncoupling protein-2 (UCP-2), which can shuffle protons over the mitochondrial membrane independent of ATP-production. This decreases the membrane potential and therefore reduces production of ROS. However, an important side effect is increased oxygen consumption (QO_2) which may result in hypoxia and nephropathy.

The aim of the present study was to investigate the regulatory effects of glutamine on the UCP-2 and thus the QO_2 in kidney mitochondria isolated from wild type and UCP-2 knockout mice. Isolated kidney cortex mitochondria were analyzed in terms of QO_2 with an Oxygraph-2k.

An increase in QO_2 in the groups treated with glutamine and was evident and there was also an increase inhibition of UCP-2 by GDP in the same groups. The combined results indicated that glutamine had a regulatory effect on mitochondrial uncoupling, possibly by UCP-2 or other isoforms.

Sammanfattning

Diabetes är en av vår tids mest utbredda sjukdomar, den påverkar miljoner av folks normala liv och sägs stå för ungefär 50 % av kostnaderna för patientvård i USA. Det finns två typer av diabetes, typ I, insulin beroende diabetes och typ II, icke-insulin beroende diabetes. Diabetes ökar produktionen av reaktiva syre former (ROS) vilket kan skada många av kroppens celler, detta kallas ofta för oxidativ stress. I njuren resulterar den ökade produktionen av ROS en aktivering av mitokondrieproteinet UCP-2 som transporterar protoner över mitokondriemembranet och sänker membranpotentialen vilket minskar risken för ROS. Denna transport har en viktig biverkning, ökad syrekonsumtion (QO_2) i njuren, vilket kan resultera i hypoxi och orsaka nefropati.

Målet med denna studie var att undersöka den regulatoriska effekten av glutamin på UCP-2 och därigenom QO_2 i isolerade mitokondrier från vildtyp och UCP-2 knockout möss. Hos de isolerade mitokondrier analyserades QO_2 med en Oxygraph-2k.

Resultaten visade en ökning i QO_2 hos grupperna som var behandlade med glutamin, inhiberingen av UCP-2 med GDP var också ökad i dessa grupper. De sammanlagda resultaten indikerade en regulatorisk effekt av glutamin på mitokondriell frikoppling, troligen via UCP-2 eller andra isoformer.

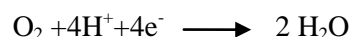
Introduction

The kidney and oxygen consumption

The kidney is responsible for removing waste material through the production of urine and to control the volume and composition of our body fluids. The kidneys produce 180 liters of primary urine per day and 99% is reabsorbed in terms of electrolytes, osmotic particles and water to maintain homeostasis. 65% of sodium and water are reabsorbed in the proximal tubule in the kidney cortex via active transportation. Because of high requirement of adenosine triphosphate (ATP) to support the active transport of electrolytes, proximal tubular epithelial cells have a high concentration of mitochondria, producing ATP through the electron transport chain (ETC) and ATP-synthase¹. The renal blood flow consists of 25% of cardiac output and the kidney extracts 10-15% of oxygen from the blood. This is in contrast to other organs with up to 45% of oxygen extraction, such as the heart^{2,3}. Despite a high perfusion, kidney cortex oxygen tension is low⁴. This can be explained by the structural design of the renal vasculature where arteries and veins run in parallel thus allowing oxygen to diffuse from the arteries to the venous system⁵. Therefore cortical tissues are at risk of developing hypoxia if the oxygen consumption (QO_2) were to increase^{5,6}.

Electron transport chain

The production of ATP occurs in the inner membrane of the mitochondria using the ETC^{2,5,6}. The ETC consists of four complexes (Fig. 1), NADH delivers electrons (e^-) to complex I and $FADH_2$ delivers electrons to complex II⁷. The electrons are then transported within the membrane using carrier molecules to complex III and then transferred from complex III to complex IV using cytochrome C via the intermembrane space^{6,7}. In complex IV the electrons react with oxygen (O_2) and protons (H^+) to create water^{6,7}.



Each time two electrons are transferred through complex I, III and IV protons are transported from the mitochondrial matrix into the intermembrane space, altogether ten protons are transported for every two electrons passing through the ETC^{6,7}. This transport of protons creates a H^+ gradient that constitutes the mitochondrial membrane potential driving the ATP-synthase⁶. For ATP production the ATP-synthase requires adenosine diphosphate (ADP), which is transported over the membrane using adenosine nucleotide transporter (ANT), translocating ADP and ATP into the intermembrane space and subsequently, the cytosol.

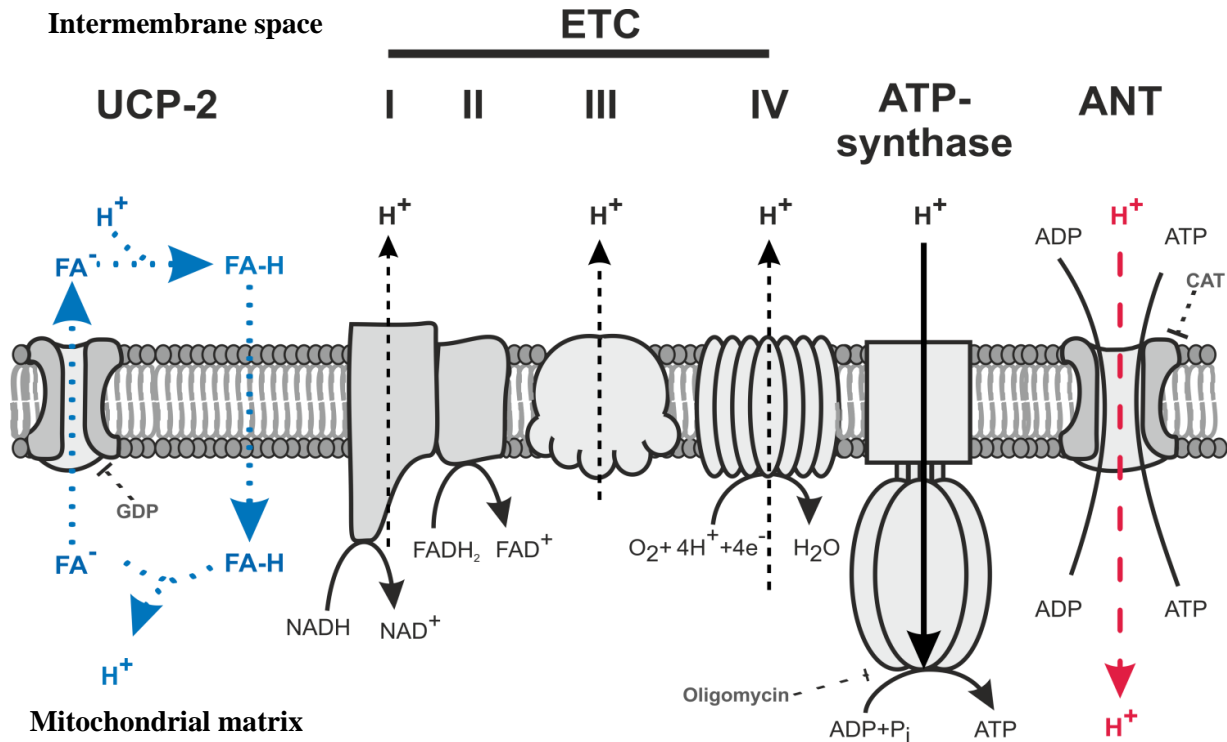
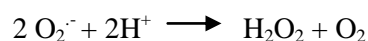


Figure 1. The electron transport chain in the kidney with inhibitors and the mechanism for uncoupling via adenosine nucleotide transporter (ANT) and uncoupling protein-2 (UCP-2). FA – Fatty acids; GDP – Guanidine diphosphate; CAT – Carboxyatractyloside; ADP – Adenosine diphosphate; ATP – Adenosine triphosphate. Modified from¹⁷.

Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) is a general term for free radicals of oxygen that include pure oxygen radicals, such as superoxide (O₂⁻) and hydroxyl (OH[•]), and non-radical oxidizing agents, such as hydrogen peroxide (H₂O₂). The most common radical is O₂⁻⁸. ROS can cause cellular damage by modifying protein, DNA and fatty acyl groups in the cellular membrane, thus interfering with normal cell functions. Damage by ROS is known as oxidative stress⁹. The body's immune system, macrophages and neutrophils, purposely generated ROS to kill pathogens and ROS play a major part in many cellular signaling pathways⁹⁻¹¹. ROS is produced in many metabolic pathways but the major source of ROS is the ETC⁹, complex I and III, which is increased by a hyperpolarized mitochondrial membrane¹².

The increased delivery of electron donating molecules such as NADH and FADH₂ causes increased transport of protons over the membrane to the point where inhibition of electron transport occurs¹². Excess electrons will be trapped in the ETC and may slip and react directly with O₂ creating O₂⁻¹³. Protection from oxidative stress is provided by, among other enzymes, superoxide dismutase (SOD)¹⁴. SOD catalyzes the reaction:



The reaction creates H_2O_2 and O_2 . H_2O_2 is significantly more stable and can easily be dismutated into water and O_2 by the enzyme catalase¹⁴. Mitochondrial production of O_2^- can also be prevented by decreasing the mitochondrial membrane potential, thus preventing hyperpolarization and inhibition of complexes I and III. This can be achieved by mitochondrial uncoupling usually performed by uncoupling proteins (UCP's), these include UCP-1 through UCP-5¹⁵.

Uncoupling Protein-2

UCP-2 is the only isoform of UCP's expressed in the kidney¹⁶. Mitochondrial uncoupling denotes shunting of protons over the inner membrane independently of ATP-production. This lowers the membrane potential and the formation of ROS is therefore decreased^{6,16-19}. UCP-2 does not by itself transport protons over the membrane, a charged fatty acid anion (FA) binds to the proton and can then diffuse across the membrane where it becomes deprotonated. UCP-2 works as a transportation channel for the FA back to the intermembrane space, this is called fatty acid cycling (Fig. 1)²⁰. The amino acid glutamine has been shown to regulate UCP-2 expression by increasing translation of UCP-2 mRNA and is for now the only amino acid known to have an regulatory effect on UCP-2 expression²¹. Increased mitochondrial uncoupling using UCP-2 come at the cost of increased oxygen consumption, this side effect may be harmful to the kidney as it may cause kidney hypoxia^{5,6}.

Diabetes Mellitus and its consequences

There are two kinds of diabetes, Type I, insulin dependent diabetes and type II, non-insulin dependent diabetes. Type I diabetes debuts at around 14-15 years of age and is caused by destruction of the insulin producing β -cells in the pancreas by an autoimmune disorder, resulting in an insufficient production of insulin. In turn, insufficient production of insulin results in decreased uptake of glucose and increased blood glucose. Excess glucose is lost in the urine and by being an osmotic particle, glucoseuria results in osmotic diuresis that may result in dehydration. Type I diabetes can also cause neural, cardiac and vascular disorders, such as stroke, heart attack and ischemia in the limbs¹. Furthermore, diabetes is known to increase QO_2 , resulting in decreased oxygen tension in the kidney that may ultimately result in hypoxia and nephropathy²²⁻²⁵. Importantly, diabetes increases UCP-2 expression which might account for increased QO_2 , resulting in hypoxia in the diabetic kidney^{18,26}.

Type II diabetes, also called insulin resistance diabetes, is caused by an increased resistance to the effects of insulin^{1,27}. Compared to type I diabetes the type II is more common, more than 90% of all the diabetes cases in the world is type II and it is most common between ages of 50 and 60. This type of diabetes does not cause all the same complications as type I, it will however cause a number of different cardiovascular diseases such as, arteriosclerosis, hypertension and organ damage due to high blood glucose levels¹. Almost 80% of type II diabetes is related to overweight and high body mass index (BMI) and it stands for almost 50% of the total health care inpatient costs in the United States²⁸.

Hypothesis and aim

The diabetic kidney has increased mitochondrial membrane potential and increased production of ROS¹⁸. Glutamine can also increase the expression of UCP-2 by increasing translation of UCP-2 mRNA²¹. Both glutamine and diabetes may cause increased expression of UCP-2 and increased uncoupling¹⁸. This can result in increased QO_2 , decreased oxygen tension and may result in hypoxia and renal damage in the kidney.

The hypothesis in the present study is that production and activation of UCP-2 in the diabetic kidney, and therefore QO_2 , can be regulated by glutamine. The aim was therefore to investigate this by using kidney cortex mitochondria from mice with induced diabetes and increase intake of glutamine to detect alterations in QO_2 . The specific role of UCP-2 was investigated using UCP-2 knockout mice.

Material and method

All chemicals were from Sigma Aldrich CO. LLC. St. Louis, USA, if nothing else is mentioned.

All solutions and isolated mitochondria were kept on ice or in 4 °C during the entire experiment.

UCP-2 knockout mice and corresponding wild-type littermates were either untreated, treated with glutamine (Dipetiven, 1mg/kg body weight, Fresenius Kabi AB, Uppsala, Sweden, 2 weeks), alloxan to induce diabetes (75 mg/kg body weight dissolved in 0.2 ml physiological saline, 2 weeks) or glutamine and alloxan in combination.

Table 1. Groups used in the present study.

Wild-type – Control	UCP-2 Knockout – Control
Wild-type – Glutamine	UCP-2 Knockout – Glutamine
Wild-type – Diabetes	UCP-2 Knockout – Diabetes
Wild-type – Glutamine + Diabetes	UCP-2 Knockout – Glutamine + Diabetes

Isolation of kidney cortex mitochondria

After cervical dislocation the kidneys were removed and placed in mitochondrial buffer A, containing (in mmol/l): 250 sucrose, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mg/ml Bovine Serum Albumine (BSA, Fraction V), pH 7.4 (controlled with KOH), 300 mOsm/kg H₂O. The kidney cortex was isolated on ice and the isolated cortex pieces were then transferred to 5 ml of new mitochondrial buffer A. The isolated cortex was then homogenized in a Potter-Elvehjem homogenizer at 600-800 rpm (4-6 strokes in total) until no visible pieces were observed. The homogenizer was kept in ice mixed with water to ensure a low temperature throughout the homogenization process. The homogenized cortex were transferred to eppendorf tubes and centrifuged, 800*g, for 10 minutes at 4 °C and the supernatant transferred to new tubes, carefully not including the buffy coat. The supernatant was then centrifuged at 8000*g for 10 minutes at 4 °C the pellets rinsed with mitochondrial buffer A and stored on ice.

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured in an Oxygraph-2k (O2k) (Oroboros Instruments Corp, Innsbruck, Austria) after calibration by air saturation.

The chamber of the O2k was filled with 2 ml of mitochondrial buffer B containing (in mmol/l): 68 sucrose, 198 mannitol, 2 EGTA, 5 MgCl_2 , 5 KPO_4^- (from a 1M mix of K_2HPO_4^- and KH_2PO_4^-), 10 HEPES, 3 mg/ml BSA, pH 7.1, 330 mOSM/kg H_2O and 20 μl of glutamate (1 M) was added to both chamber A (control chamber) and chamber B (inhibitor chamber). The saturated fatty acid palmitate 48 $\mu\text{mol/l}$ (2.3 $\mu\text{g/ml}$) and the ATP-synthase inhibitor oligomycin were also added to chamber B before the addition of mitochondria. The mitochondria pellet was dissolved in 200 μl of mitochondrial buffer A and 100 μl of suspended mitochondria was added to each chamber. After stable QO_2 was achieved 20 μl ADP (30 mM in 10 mM HEPES, 0.6mol MgCl_2 /mol ADP) was added to chamber A, to achieve maximal respiration and calculate respiratory control ratio (RCR). In chamber B 20 μl Guanosine 5'-diphosphate (GDP, 4.4 mg Guanosine 5'-diphosphate sodium salt dissolved in 200 ml mitochondrial buffer B) was added and thereafter 2 μl Carboxyatractyloside (CAT, 2 mg Carboxyatractyloside potassium salt dissolved in 444 μl water) was inserted. Analysis of QO_2 was made using DATALAB 4 software (Oroboros instruments Corp, Innsbruck, Austria).

Respiratory control ratio (RCR) was calculated by dividing the QO_2 after the addition of ADP with the QO_2 before ADP. RCR is indicative of mitochondrial health and a $\text{RCR} \geq 4$ is required before performing additional experiments. A sample was taken from each chamber to correct QO_2 for protein content using DC Protein Assay (Bio-Rad Laboratories, CA, USA).

Results

There was a tendency to increased mitochondrial QO_2 in wild-type groups after glutamine treatment but the UCP-2 knockout groups did show the same tendency (Fig. 2.). There was a tendency of increased inhibition by GDP, thus decreasing QO_2 , in the wild-type groups that were treated with glutamine. The UCP-2 knockout groups did also show this tendency, however not as strong (Fig. 3.) There was a decrease in QO_2 in the wild-type group that had both alloxan and glutamine treatment when CAT was added (Fig. 4.). All the groups had a RCR above 4 (Fig. 5.).

Mitochondria Oxygen consumption

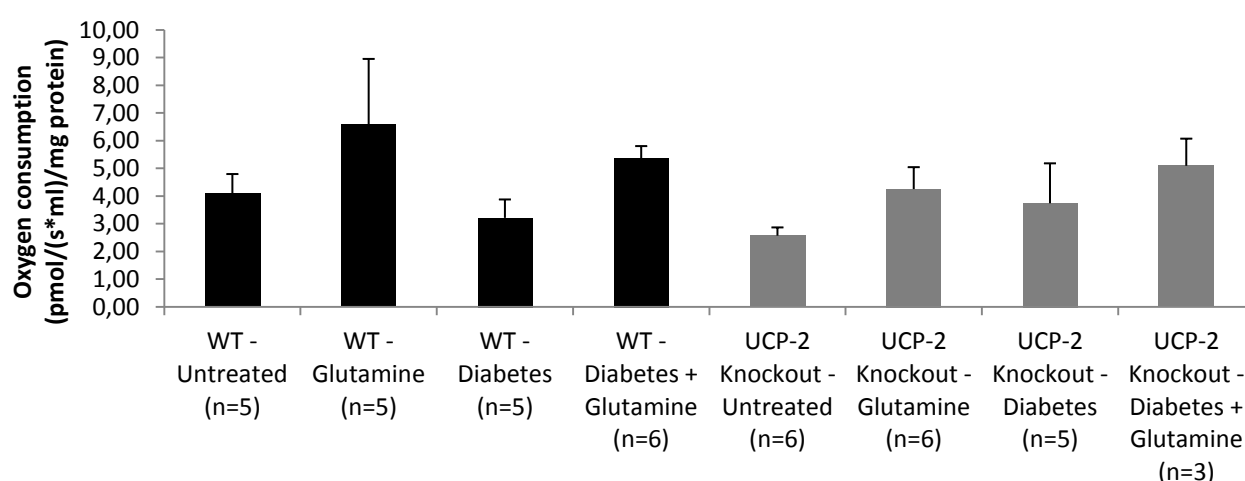


Figure 2. Baseline mitochondrial oxygen consumption for all the groups. All groups have ATP-synthase inhibited by oligomycin. All values are presented as mean \pm SEM.

Oxygen consumption decrease after GDP

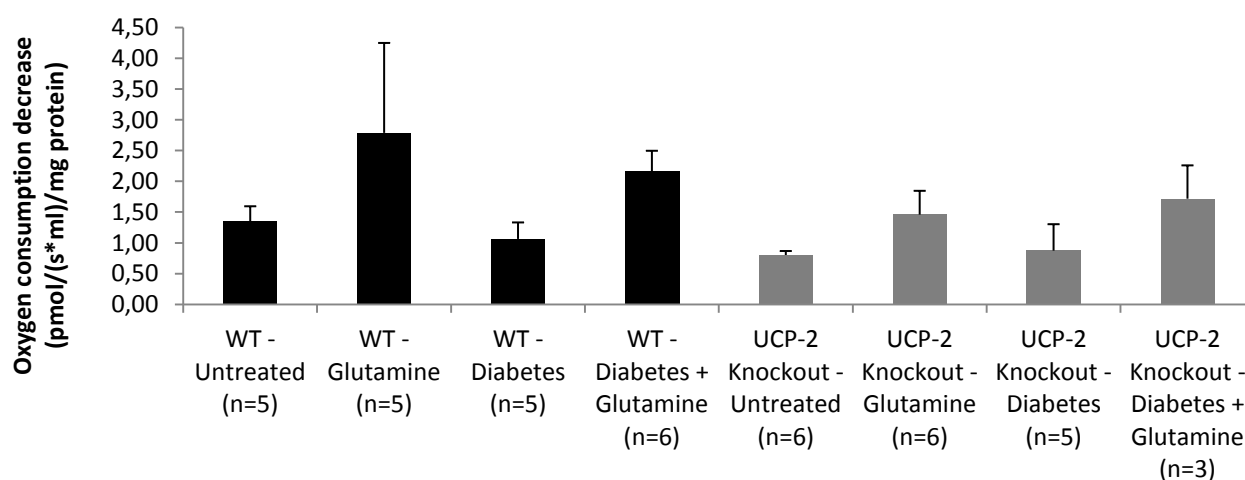


Figure 3. Decrease in mitochondrial oxygen consumption after addition of GDP, an inhibitor of UCP-2. All values are presented as mean \pm SEM.

Oxygen consumption decrease after CAT

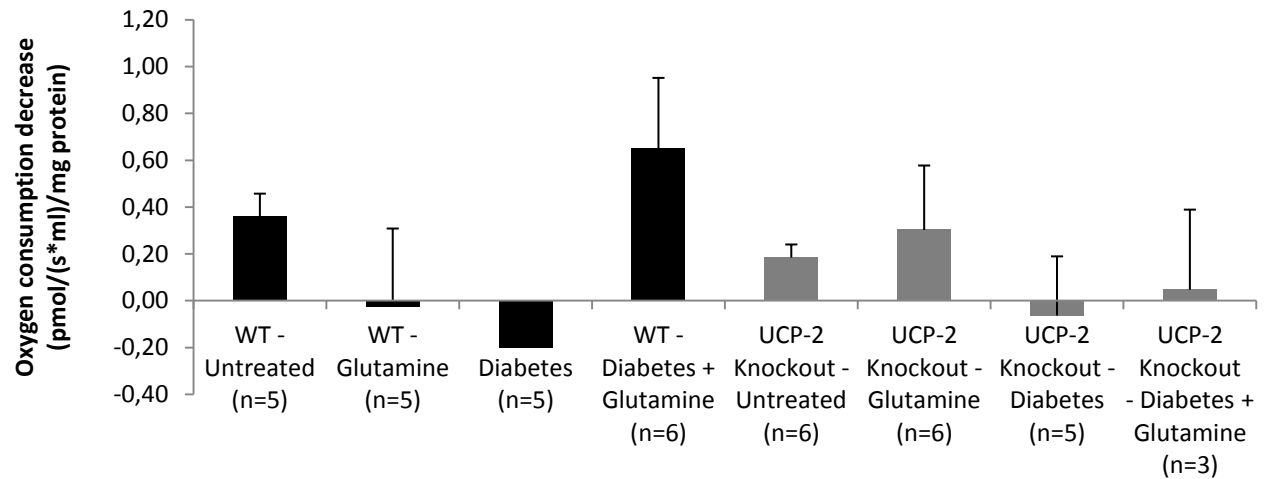


Figure 4. Decrease in mitochondrial oxygen consumption after addition of CAT, an inhibitor of ANT. All values are presented as mean \pm SEM.

Respiratory control ratio (RCR)

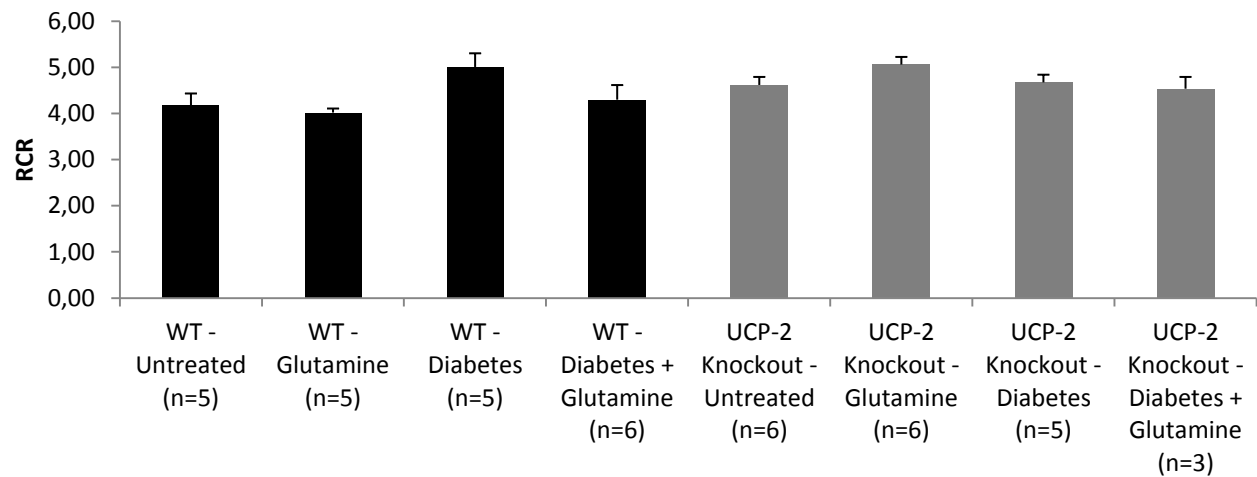


Figure 5. Respiratory control ratio (RCR) for all groups. All values are presented as mean \pm SEM.

Discussion

The major finding of the present study is that mitochondrial QO_2 can be affected with glutamine in wild type mice. There was also a small increase in QO_2 in the UCP-2 knockout groups after glutamine treatment. It's possible that other UCP-isoforms, UCP-4 and UCP-5 which is also expressed in mouse kidney²⁹, may be involved in regulation mitochondrial QO_2 . It is known that diabetes can increase expression of UCP-2 in the kidney²⁶ and increasing amounts of NADH and $FADH_2$ sequentially increase the O_2 required for electron transport⁶. This increasing QO_2 may result in hypoxia^{5,6}. The results demonstrated increased QO_2 in the animals treated with glutamine. However, diabetes alone did not affect QO_2 in wild-type, which was unexpected since diabetes increases QO_2 in rats²⁶. It may be postulated that UCP-2 may not be activated in mouse kidneys. Further measurements of UCP-2 protein excretion will clarify this issue. Since wild type groups treated with glutamine displayed a greater increase in QO_2 , it may be postulated that the increased QO_2 is caused by increased uncoupling by UCP-2 and that is activated and regulated by glutamine. This is supported by increased inhibition by GDP, a known inhibitor of UCP's³⁰, in the same groups. However, these effects were also evident in UCP-2 knockout mice, indicating that mitochondrial QO_2 is directly regulated by glutamine. It cannot be excluded that glutamine may regulate other UCP-isoforms that is present in mouse kidney.

Furthermore, an increased ANT uncoupling was evident in diabetic wild-type treated with glutamine, indicating that glutamine might have regulatory effects on ANT uncoupling during increased stress caused by diabetes and increased glutamine intake. ANT is known for its ability to replace UCP-2's functions if UCP-2 function is severely reduced¹⁸. However, interestingly, similar effects by CAT were not evident in UCP-2 knockout mice. Additional studies are warranted due to interesting tendencies before making further conclusions.

Conclusions

The present study concludes that glutamine can activate and regulate uncoupling through UCP-2, resulting in increased QO_2 . This finding is supported by an increased inhibition by GDP.

Future aspects

The present study revealed some interesting tendencies that need to be thoroughly investigated. The study only featured 5-6 mice in each group and thus, needs to be increased with added experiments. Future studies should also investigate the effect of glutamine on ANT and it's uncoupling in mitochondria. Furthermore the possible role of other UCP-isoforms in mediating observed effects needs to be investigated.

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