The role of UCP-2 in the onset of diabetic nephropathy – a regulatory mechanism via glutamine

Caroline Granberg

Department of Medical Cell Biology, division of integrative physiology

Supervisor: Malou Friederich-Persson, PhD
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Abstract

One third of all diabetic patients develop kidney damage, i.e diabetic nephropathy. Currently there is no curative treatment and ultimately results in dialysis, transplantation, constituting a major and costly health problem. Diabetic nephropathy is also closely associated with increased mortality.

The mitochondrion is the energy-producing compartment of the cell but also an important source of reactive oxygen species (ROS) resulting in oxidative stress. Oxidative stress is detrimental to the cells, resulting in DNA damage, altered enzyme function, lipid peroxidation and ultimately, apoptosis. The mitochondria can protect itself from increased ROS production via a mitochondrial protein, uncoupling protein 2 (UCP-2). UCP-2 can diminish the mitochondria membrane potential leading to a decreased ROS-production. Interestingly, UCP-2 is up regulated in diabetic patients and animal models of diabetes. Though uncoupling via UCP-2 is a mechanism to decrease ROS formation, uncoupling come at the cost of increased oxygen consumption. The kidney is therefore at risk of develop hypoxia, an acknowledged pathway to nephropathy. Studies have demonstrated that the amino acid glutamine can increase the level of kidney damage in diabetes via UCP-2-mediated uncoupling.

The aim of the present study was to investigate the role of glutamine in inducing kidney damage in normoglycemic and diabetic mice. Furthermore the role of UCP-2 was investigated using genetically modified mice lacking UCP-2. Proteinuria, a clinically established sign of nephropathy was only affected by glutamine in diabetic wildtype mice and remained unaffected in all other groups. This indicates that glutamine may indeed induce nephropathy through a UCP-2 dependent mechanism.
Sammanfattning

En tredjedel av alla patienter med diabetes utvecklar njurskador, d.v.s. diabetes nefropati. För närvarande finns ingen botande behandling och detta leder till dialys och transplantation, som utgör ett stort och kostsamt hälsoproblem. Diabetes nefropati är också relaterat till en ökad dödlighet.

**Introduction**

**The kidney**

The kidney regulates body homeostasis and produce urine by controlling secretion and reabsorption of electrolytes and water, affecting acid-base balance, regulating arterial pressure and removal of metabolic waste products and exogenous substances\(^1\).

Each kidney contains approximately one million functional units, nephrons. Each nephron consists of the glomerulus, a bundle of capillaries encapsulated in Bowman’s capsule in which filtration occurs. The filtrate is then subjected to absorption and/or secretion along tubule, converting the filtrate into secondary urine that enters the pelvis of the kidney and the urinary tracts. The long tubule is divided into the proximal tubule, the loop of Henle, macula densa, distal tubule, connecting tubule, cortical collecting tubule, medullary collecting tubule and the collecting duct. The loop of Henle together with the medullary collecting tubule and the collecting duct is located in the renal medulla while the rest is located in the renal cortex\(^1\).

The kidney is highly perfused with approximately 22% of the cardiac output reaching the kidneys. Despite a high blood flow the kidney oxygen tension is quite low rendering the kidney vulnerable to alterations in oxygen availability\(^1\).

**The mitochondrion**

The mitochondria are the energy-producing unit of the cell consisting of an outer and an inner membrane. In the mitochondrial inner membrane cellular energy is produced from a series of complexes called the electron transport chain (ETC). The ETC consists of four complexes, an ATP-synthase and an adenosine nucleotid transporter (ANT). Electrons are shuffled through the complexes to complex IV where a reduction of oxygen occurs, resulting in water. Simultaneous as electron transfer hydrogen ions (H\(^+\)) are translocated into the intermembrane space (IMS), generating membrane potential which the adenosine triphosphate (ATP)-synthase uses to produce ATP. This chain of event is called oxidative phosphorylation. ATP is then transported out of the mitochondria to the cytoplasm by the ANT\(^2\).
**Oxidative stress**

The mitochondrion is not only responsible for the ATP production but is also an important source of reactive oxygen species (ROS). The electrons in the ETC can react directly with oxygen, thereby forming superoxide, a highly reactive ROS. This occurs normally in the cells although the levels of ROS are kept low in a healthy cell via antioxidant defense system. Other ROS are hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (OH$^-$). Although ROS is also known as a cellular signaling system an increased formation of ROS have dire consequences for the cells in terms of enzyme dysfunction, DNA mutations and cellular membrane damages, ultimately resulting in cellular death, apoptosis$^3$.

**Diabetes mellitus**

Diabetes mellitus is a disease characterized by hyperglycemia in the blood existing in two different types. Type 1, an autoimmune disease, debuts in a young age after the β cells of the pancreas is destroyed, resulting in an insufficient amount of insulin. Type 2, strongly associated with lifestyle and obesity$^4$ (defined as body mass index greater than or equal to 30 kg/m$^2$)$^5$, commonly debuts at an older age and the cause is peripheral insulin resistance, resulting in hyperglycemia. Type 2 diabetes, like many other chronic diseases, has a prolonged asymptomatic phase but when the symptoms occurs, they appear as increased thirst, polyuria, frequent urination, weight loss and fatigue, as for type 1$^6,7$. 
**The kidney and hyperglycemia**

One third of all diabetic patients are suffering from complications in the kidney, called diabetic nephropathy. Today in the western countries, diabetes nephropathy is the leading cause chronic kidney disease (CKD) and end-stage renal disease (ESRD). Diabetic nephropathy is progressive and has no curative treatment, leading to dialysis and/or transplantation.

The main indicator of kidney damage is proteinuria. Other common used markers for kidney function is a reduced estimated glomerular filtration rate (eGFR), subdividing the severity of CKD into five stages, and an increased serum creatinine level. A reduced function of the kidney increases the risk of ESRD. CKD classification system is based mainly of degrees in eGFR reduction and 1-5 is defined as eGFR >90, 60-89, 30-59, 15-29 and <15 ml/min/1.73 m² respectively. Furthermore the findings should be consistent for 3 month or more. Findings in stage 1-2 in terms of albuminuria, hematuria or sonographic abnormalities are also required.

**Oxidative stress in the diabetic kidney**

It is known that the oxidative stress in the diabetic kidney is increased due to increased production of ROS, mostly superoxide, that is generated by the mitochondria. More superoxide is produced due to an increased membrane potential in the mitochondria leading to a reduced movement of the electrons, that in turns leads to the increased risk of electrons slipping to oxygen directly instead of moving forward in the complexes to form water. An antioxidant defense against increased mitochondria superoxide production is mitochondria uncoupling.
Mitochondria uncoupling via uncoupling protein 2 (UCP-2)

Mitochondria uncoupling is the process of releasing H\(^+\) independently of ATP-production and is usually performed by UCP 1-5. UCP-1 was the first characterized in brown adipose tissue (BAT) where its sole purpose is to produce heat. UCP-1 is of importance in hibernating animals and small infants. UCP-3 is expressed in skeleton muscle and involved in regulating mitochondrial oxidative stress levels. UCP-4 and 5 is ubiquously expressed solely in the brain and their function is largely unknown. UCP-2 is ubiquously expressed in tissues such as skeleton muscle, liver, pancreas and the kidney\(^{19,20}\). Studies have demonstrated that UCP-2 increases in diabetic animal models, resulting in increased kidney oxygen consumption. Since mitochondria uncoupling increases oxygen consumption an increased uncoupling may lead to chronic hypoxia in the kidney\(^{21}\).

In diabetic mitochondria, the passage of electrons in the ETC are increased which leads to the up-regulation of the membrane potential and therefore the increased formation of ROS\(^{22}\). Therefore the cell has developed a mechanism to protect it self from this unfavorable out come in the diabetic kidney and that is the shuffling of protons from the intermembrane space (IMS) to the matrix via UCP-2, independent of the ATP production. Although this is an important way to limit the superoxide production, the oxygen consumption increases which may be detrimental for the kidney\(^{23}\). If the UCP-2 is knocked down by small interference (si)RNA the adenine nucleotide translocator (ANT) takes over and shuffles protons from the IMS to the matrix instead of UCP-2, demonstrating that mitochondria uncoupling is an important mechanism in diabetic kidneys\(^{21}\).
Glutamine and diabetes

Glutamine is a free amino acid in the circulation and the precursor to the antioxidant glutathione\(^{24,25}\). Studies have demonstrated that glutamine has immunomodulating properties and therefore suppresses inflammatory responses\(^{25}\). It has been pointed out that glutamine also has the ability to decrease oxidative stress in diabetes due to its antioxidant effect\(^{26}\). Although glutamine has an antioxidant effect, studies have reported that glutamine supplementation has contributed to renal injuries in diabetic rats but not in normoglycemic rats\(^{27}\). Furthermore, other studies have demonstrated the effect of glutamine on UCP-2 expression. C. Hurtaud et.al demonstrated that an increase in glutamine correlates with an increase in UCP-2 and glutamine is the regulating factor, i.e glutamine stimulates UCP-2mRNA translation\(^{28}\). In accordance to these studies it is reasonable to assume that glutamine may contribute to an increased level of nephropathy in diabetic patients and animal models, due to the damages caused by the up regulation of UCP-2.

Aims

The present study aimed to investigate if glutamine supplementation induces nephropathy in normoglycemic and diabetic mice compared to corresponding control animals. Furthermore it aimed to investigate the specific role of UCP-2 in mediating observed effects using genetically modified mice lacking UCP-2.
Materials and methods

Animals and chemicals

All chemicals were from Sigma-Aldrich, St. Luis, MO unless otherwise stated. All mice (UCP-2 knockout mice with C57/Bl6 background and corresponding wildtype littermates) had free access to tap water and standard mouse chow and were housed in a light and temperature controlled environment. The Uppsala animal ethics committee approved all animal experiments. Four groups received glutamine (1 mg/kg b.w., Dipetiven Fresenius Kabi AB, Uppsala, Sweden) for two weeks before the terminal experiments. Four groups received injections of Alloxan, 75 mg/kg b.w. intravenously diluted in 0.2 ml physiological saline, to induce diabetes mellitus two weeks before the terminal experiments.

The following groups were:

- Wildtype (n=6)
- Wildtype+glutamine (n=5)
- Wildtype+diabetes (n=6)
- Wildtype+diabetes+glutamine (n=6)
- UCP<sup>-/-</sup> (n=6)
- UCP<sup>-/-</sup>+glutamine (n=6)
- UCP<sup>-/-</sup>+diabetes (n=5)
- UCP<sup>-/-</sup>+diabetes+glutamine (n=3)

Metabolic chamber

Mice were kept in metabolic chambers (Harvard apparatus, England, United Kingdom) for 16 hours (Figure 1) and potassium-, sodium-, protein excretion, food and water intake, urine and faeces excretion and blood glucose, osmolality and body weight were measured. The mice were provided food and water.
Determination of proteins, potassium, sodium, osmolality and creatinine

In collected urine, urinary protein was determined by DC Protein Assay (Bio-Rad Laboratories, CA, USA), urinary potassium and sodium excretion were determined by flame photometry (IL943, Instrumentation Laboratory, Milan, Italy), urine osmolality by the Fiske micro-osmometer (Fiske Associates, Massachusetts, USA) and creatinine was determined by Creatinine (Enzymatic) kit (Abbott Laboratories, Illinois, USA), according to manufacturer’s instructions.

Figure 1. Metabolic chamber used in the present study. The mice were provided with food and water on the sides and on the bottom there was a urine collecting vessel.
Results

Body weight did not differ among the groups (Fig 10). Diabetic animals displayed increased blood glucose levels compared to normoglycemic animals, but was not affected by glutamine treatment or UCP-2 deletion (Fig 11). Food and water intake did not differ among the groups (Fig 6 and 7) and neither did urine or faeces excretion (Fig 8 and 9). There were no significant differences in osmolality, potassium- and sodium excretion between the groups (Fig 5, 4 and 3). Urinary protein excretion was increased in diabetic wildtype group with glutamine supplementation but did not differ among the other groups.

Figure 2. Urinary protein excretion during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2−/−) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
Figure 3. Sodium excretion during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2−/−) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.

Figure 4. Potassium excretion during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2−/−) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
**Figure 5.** Osmolality during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2\(^{-/-}\)) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.

**Figure 6.** Food intake during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2\(^{-/-}\)) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
**Figure 7.** Water intake during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2\(^{-}\)) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.

**Figure 8.** Urine excretion during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2\(^{-}\)) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
**Figure 9.** Faeces excretion during 24 hours in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2-/-) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.

**Figure 10.** Body weight in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2-/-) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
Figure 11. Body weight in wildtype mice (Wt) and UCP-2-knockout mice (UCP-2−/−) during normoglycemic conditions or after diabetes induction (DM) with or without glutamine supplementation (G). All values are presented as mean ±SEM.
Discussion

The main finding in the present study was increased protein excretion in wildtype diabetic mice supplemented with glutamine. This only occurred in diabetic mice and importantly, not in diabetic mice lacking UCP-2. Proteinuria is a clinically established sign of nephropathy, therefore indicating kidney damage in these animals. The possible mechanism may include increased uncoupling via UCP-2 as glutamine is known to transcriptionally control the level of UCP-2\(^2^8\). Interestingly, increased kidney damage after glutamine supplementation to diabetic rats has previously been demonstrated by others\(^2^7\). Tatiana et al. induced diabetes in male Wistar rats by intravenous injection of 65 mg/kg b.w. streptozotocin (STZ) dissolved in citrate buffer. The diabetic state was confirmed 48 hours later by blood glucose level above 200 mg/dl. Glutamine supplementation was given as an aqueous solution by gavage once a day for 15 days. Expression of important pro-inflammatory interleukins IL-6 and IL-1 beta and blood creatinine was measured and the area of glomeruli was observed. IL-6 and IL-1 beta increased and total area of the glomeruli was decreased by 10% in diabetic rats treated with glutamine and blood creatinine, indicating glutamine may indeed induce renal injuries.

Increased mitochondria uncoupling via UCP-2 is previously known in diabetic kidneys\(^2^3\) and may indeed contribute to nephropathy via increased oxygen consumption. Importantly, increased oxygen consumption may be detrimental for the diabetic kidney as it may result in kidney tissue hypoxia, resulting in nephropathy.

Friedrich-Persson et al demonstrated that increased mitochondria uncoupling results in kidney tissue hypoxia and proteinuria\(^3^0\). Levels of proteinuria and developing hypoxia have been demonstrated to correlate with a more rapid loss of kidney function in patients suffering from CKD\(^3^1\). As UCP-2-mediated uncoupling results in increased oxygen consumption, glutamine control of UCP-2 level may be important in the development of nephropathy.

It was surprising that Na\(^+\)/K\(^+\)/osmolality excretion was not different among the groups as diabetic animal models normally have increased food intake and therefore increased secretion. However, a methodological problem in the present study was the design of the metabolic chambers. Some of the urine that was supposed to end up in the collecting vessel on the bottom of the metabolic chamber, instead ended up on the
sides of the vessel, making the urine volume quite unreliable. One thing to keep in mind when using metabolic chamber is how the cages are stored while the mice resides in the cage. When the mice are close and can see one another, they may easily become stressed from the near presence of another mouse and therefore the measurements may reflect non-physiological conditions. Therefore, the cages should be stored separately or covered at the transparent parts where the mice can see through the cage, shielding the mice from the presence of others. Importantly, this was performed in the present study.

Another important factor to consider is that mice should have the chance to get used to the metabolic chamber in a separate run before the experiment is conducted. It may indeed be a good idea to make the cage more comfortable for the mice, for example inserting some kind of house for the mice to feel assured in and also make sure that the mice are not cold by housing the cages in a temperature-controlled environment.

It is uncertain why most of the mice did not eat or drink, though the measurements could be erroneous due to the design of the metabolic chamber. The values that can be seen is also unreliable because some of the mice scribbled away some of the food to the bottom of the metabolic chamber and there is even a possibility that the mice urinated in the food which would lead to fallacious data. Furthermore urine and faeces data is unreliable because all of the mice urinated outside of the urine-collecting vessel.
Conclusion

The diabetic wildtype mice receiving glutamine in their drinking water displayed increased kidney damages than other groups. Importantly, this was evident only in wildtype mice and not in mice lacking UCP-2, demonstrating a pivotal role of UCP-2 in mediating the observed effects. Therefore, it is concluded that glutamine induces nephropathy via an UCP-2 dependent mechanism. Further studies are warranted.

Perspectives and future studies

To further strengthen the conclusions additional experiments should be performed. One thing to consider is the length of the period for the treatments, both glutamine and diabetes. It could be that two weeks are not sufficient to induce renal damages, in untreated diabetic animals. Furthermore, as C57/Bl6 are known to be quite resistant to develop nephropathy a change of the mouse strain might be considered. The last thing to consider is the dose. A higher dose may have provided a different outcome.

References


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