Differential effects of NO and HNO of rat kidney cortex mitochondria, regulation under physiological oxygen tension

Mona Nouhi
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Abstract

The effects of nitric oxide (NO) on mitochondria from different organs of the body, including kidney has been studied for some years, and today it is known that low concentrations of NO are important for regulating different functions in our body. On the other hand, high concentrations of NO can lead to cell death [1]. It has been demonstrated that NO can inhibit the mitochondria function under low oxygen concentration by binding to cytochrome c oxidase (complex IV) [2]. As NO binds to complex IV and deoxidizes, nitroxy (HNO) can be produced. HNO may lead to more damage by inhibiting the mitochondria, resulting in increased oxidative stress and reduced cellular availability of adenosine diphosphate (ADP) [3]. The effect of nitroxyl on mitochondria in general but especially on the kidney mitochondria is a new field for researchers. The present study project investigates how NO and HNO can affect rat kidney mitochondria under normal and low oxygen concentrations.

Corresponding author: Mona Nouhi

Mona_no@hotmail.com

Department of Medical Cell Biology
Division of Integrative Physiology
Uppsala University, Sweden

Project supervisor: Malou Friederich

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Introduction

Mitochondria are the energy producing factories in our body. In order to be able to generate energy in the form of adenosine triphosphate (ATP), many different substances must be present and various parts of the mitochondria are in focus. Mitochondria are spherical organelles in the cytoplasm of the cells and composed of an outer and inner membrane. The space between the two membranes is denoted the intermembrane space. The inner membrane of the mitochondria is large and has a folding form called cristae, the inner part of the mitochondria is denoted the matrix, a gel-like solution consisting of proteins and other substances needed for ATP synthesis such as ADP and inorganic phosphate (Pi).

The part of the mitochondria whose main task is to produce ATP is the electron transport chain (ETC), located in the inner membrane of the mitochondria. ETC includes five different protein complexes which together work to maintain a proton gradient between the intermembrane space and matrix. Energy from the proton gradient is used by complex V (ATP synthase) for an oxygen-dependent ATP synthesis called oxidative phosphorylation.

![Figure 1. Structure of a mitochondrion in electron microscopy picture (top) and in a schematic view (bottom).](image)

In the electron transport chain, each complex transfers electrons in order to maintain a proton gradient over the inner membrane. For transferring electrons between the different complexes, a variety of molecules like flavin mononucleotide (FMN), cytochrome c, Fe-S
centers and ubiquinone (Coenzyme Q, CoQ) work at the same time among the complexes. NADH and FMNH₂ are responsible for delivering electrons to complex I and II and are products from the triacarboxylic acid cycle (TCA). During an aerobic metabolism in the matrix, acetyl-coenzyme A (Ac-CoA) is produced from the oxidation of pyruvate. Later, Ac-CoA enters the TCA cycle and there generates NADH and FADH₂. At complex one NADH donates two electrons to FMN producing FMNH₂ and the electrons are transferred to the next transporter, the Fe-S center and the ultimately to ubiquinone. Ubiquinone delivers electrons both from complex I and II to complex III (cytochrome c reductase). Electrons from complex III are transferred by cytochrome c to complex four or cytochrome IV (cytochrome c oxidase). Complex IV contains a heme group that reduces molecular oxygen to water. The transferring of electrons from complex I to complex IV induces a translocation of protons from the matrix to the intermembrane space, creates an electrical and pH dependent gradient across the mitochondria inner membrane, with more positive charges and lower pH in the intermembrane space. This is known as the mitochondria membrane potential and gives off a free energy of 52.58 kcal. The energy needed for phosphorylation of ADP to ATP by complex V (ATP-synthase) is 7.3 kcal/mol/ATP. As a result the ATP synthase will consume the energy from the membrane potential for the synthesis of ATP from ADP and Pₗ. Oxidative phosphorylation produces approximately 30 ATP from one glucose molecule (Malou 2009). Several factors regulate the oxidative phosphorylation: supply of NADH and FADH₂, ADP, O₂ availability and the membrane gradient.

Figure 2. The electron transport chain (ETC) in the mitochondria inner membrane. Dashed arrows denote electron donating substrates and dashed T-symbols denotes inhibitors of mitochondria complexes. (Modified from Friedrich et al. 2009).
The mitochondria used in this project have been isolated from the kidney cortex. As one of the most central organs in the urinary system, kidneys can produce urine containing waste products and at the same time regulate the extracellular fluid (ECF), blood pressure and electrolyte balance.

Kidneys have two main parts, the cortex and the medulla, which are composed of functional tubular structures called nephrons. Each nephron is made of two main sections, the glomerulus and the tubular system. The inner part or the medulla is composed of several pyramidal structures, the medullary pyramids and has a parallel arrangement of different parts of nephrons; the loops of Henle, medullary collecting ducts and blood vessels providing medulla. Additional parts of nephrons; the proximal tubule, the cortical collecting duct, the distal convoluted tubule and the juxtaglomerular apparatus surrounded by Bowman’s capsule are located in the cortex.

As mentioned, the first step in urine production is the filtration of plasma by glomerulus, yielding primary urine without any proteins. Next, from the primary urine ions, water, glucose and urea are reabsorbed to the capillary blood. Organic substances like ammonia and H⁺ are transported to the tubular system for excretion in the urine.

The first part of the tubular system, which is located directly after glomerulus, is the proximal tubule. This tubule is made of cuboidal epithelial cells containing high amounts of mitochondria compared to other parts of the tubular system and this is the justification to the use of kidney cortex in the present study. This part is responsible for a selective reabsorption of filtered glucose, water, amino acids and ions like Na⁺, K⁺, Ca²⁺ and Cl⁻. This reabsorption is secondary active, meaning that ATP is needed. Na⁺/K⁺-ATPase (NKA) is placed on the basolateral side of the cell toward the blood vessels and pumps three Na⁺ (which

Figure 3. Anatomy of the kidney.  
Figure 4. Structure of a cortical nephron.
enters the cell through a passive mechanism from the apical membrane along with glucose) out of the cell to the circulation and two K⁺ into the cell as it hydrolyses ATP, keeping the intracellular concentration of Na⁺ low. This transport of Na⁺ to the blood establishes an osmotic gradient which is used for reabsorption of water. Thus the proximal tubule reabsorbs about 70% of Na⁺, K⁺ and 100% of filtered glucose. As mentioned, the Na⁺ transport occurring throughout the tubular system and not only in the proximal tubule are ATP dependent, meaning that kidneys must have a high production of ATP and are therefore in great need of oxygen. Indeed, the kidneys are responsible for almost 10% of total O₂ consumption in our body. The kidneys and their function are commonly affected in various diseases, such as hypertension and importantly; diabetes mellitus.

Type 1 and 2 diabetes mellitus are widespread diseases with the common hallmark of hyperglycemia as a metabolic abnormality. In type 1 diabetes elevated levels of glucose arise due to nonfunctional β cells of pancreas caused by an autoimmune reaction leading to almost no insulin production. Therefore, type 1 diabetes is an insulin-dependent disease, affecting individuals early in life before or during puberty. Patients suffering from diabetes type 1 have symptoms of fatigue, weight loss, polyuria (frequent urination) and polydipsia (extreme thirst). Ketoacidosis (high levels of ketone bodies leading to acidos) is another symptom as a result of increased mobilization of fatty acids among these patients which can also explain their weight loss.

Type 2 diabetes mellitus is a noninsulin-dependent disorder but yet with hyperglycemia. Hyperglycemia in type 2 diabetes is more complex because patients have normal β cells and even an increased insulin production but cells (mainly skeletal muscle) lose their sensitivity for insulin, resulting in high levels of blood glucose. As a result from an insulin resistance pancreatic β-cells dysfunction develops over time [4]. It must be mentioned that dysfunctional mitochondria bioenergetics has been indicated as a potential mechanism in the development of insulin resistance and type 2 diabetes [5]. Type 2 diabetes debuts later in life and insulin resistance develop slowly, often in combination with overweight (obesitas), hypertension and hyperlipidemia. Taken together, these factors are known as the metabolic syndrome and one of the best treatments is life-style changes such as diet and exercise.

Secondary complications such as retinopathy, neuropathy and nephropathy might develop eventually; approximately 30% of patients are afflicted. This is true for individuals with both types of diabetes, even if hyperglycemia is under good control. However it is evident that development of complications highly depends on the degree of the hyperglycemia [6].

One of the organs in high risk of failure is the kidneys. It has been shown that glomerular filtration rate (GFR) increases by about 30% in untreated diabetic rats and at the same time oxygen tension decreases along with an increase in oxygen consumption, which is associated with diabetic nephropathy [6]. When GFR increases it results in increased work for the
kidneys in order to reabsorb increased amounts of glucose and thereby they are in greater need of oxygen for ATP production by mitochondria. However the oxygen tension decreases and is obviously not sufficient. A possible reason for the increased oxygen consumption seen in diabetic kidneys is a decrease in inhibitory effect of nitric oxide (NO) on the oxygen metabolism in renal proximal tubule. NO-levels are decreased in diabetic kidneys and this is likely to be an effect of reactive oxygen species (ROS), degrading NO into harmful metabolites [7]. Commonly known is the fact that ROS production increases along with long term hyperglycemia.

NO in mammalian cells is synthesized by four isoforms of nitric oxide synthase (NOS), endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). NOS synthesizes nitric oxide in a two step process, first the terminal guanodino-nitrogen on the substrate L-arginine is oxidized to N-hydroxy-L-arginine, after which O₂ and five e⁻ (from NADPH) are used to produce NO and L-citrulline [5].

NO produced from nNOS acts as a diffusible neurotransmitter at synapse [8], as an intracellular messenger [9], regulates smooth muscle relaxation [10] and regulates vascular tone [5]. Inhibition of NOS therefore decreases renal blood perfusion in cortex and medulla and at the same time increases mean arterial pressure [7]. In addition NO has been associated with diabetic retinopathy, because it is shown that cataract lenses from diabetic patients have increased levels of end products from degradation of NO [5]. Many of mentioned effects of NO, is mediated through binding and activating the soluble guanylate cyclase (sGC) as its primary receptor. NO by binding to sGC, catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP) and high levels of intracellular cGMP leads to smooth muscle relaxation [10].

Interestingly, it has been shown that mitochondria may contain a special isoform of NOS, called mitochondrial NO synthase (mtNOS) which produces NO. mtNOS is activated upon stimulation of calcium, which is released from endoplasmic reticulum (ER) and enters the mitochondria through an electrogenic uniporter [8]. Activated mtNOS produces NO that by binding to ferrous heme site of cytochrome c oxidase (complex IV) of the mitochondrial ETC reversibly inhibits oxygen consumption and thereby regulate intracellular respiration rate resulting in decreased ATP levels [9]. NO and O₂ compete for the same binding site at complex IV and NO inhibition of cytochrome c oxidase is potentiated at low oxygen tensions (pO₂) or hypoxia. This is attributed to a higher affinity of NO than O₂ to complex IV. However, even with a high affinity NO cannot bind to complex IV and does not have distinguished effects under normal oxygen tensions [5].
Figure 5. NO synthesis by nitric oxide synthase from L-arginine, resulting in NO and citrulline.

As mentioned earlier, long term hyperglycemia is associated with increased levels of ROS generated by mitochondria [4]. It is believed that exposing cells to elevated levels of glucose and free fatty acids increases production of mitochondrial ROS that may destroy pancreatic β cells and contribute to the progress of secondary complications such as diabetic nephropathy [4]. Additionally, ROS and NO can react together, yielding peroxinitrite (ONOO⁻) [11]. ONOO⁻ is known to cause oxidative damage, irreversible inhibition of complex I,II and III and influences the pathogenesis of arteriosclerosis, arthritis and sepsis [5]. Another reaction involving NO that is less studied is the reducing reaction of this molecule; forming nitroxyl (HNO) [12].

HNO is one of the least studied nitrogen oxides and thus far there is no conclusive evidence for endogenous generation of HNO in mammalian cells [12]. However it has been shown that HNO may be generated in vivo, through oxidative degradation of an intermediate in NO synthesis cycle, the N-hydroxy-L-arginine (NOHA). HNO may be produced from NOHA. When NOS is deplete of one of its prosthetic groups, tetrahydrobiopterin [12] and also via the interaction of NO with ETC in mitochondria, especially through the reaction with cytochrome c and ubiquinol [12]. Since there is no conclusive evidence supporting the fact that HNO can be generated in mammalian cells, there is no data about effects of HNO in vivo. A recent study has reported that HNO can be a potent vasodilator [12]. And HNO displays beneficial effects in ischemia reperfusion injury, if administered before reperfusion [1]. Furthermore HNO has been shown to be able to inhibit the action of Poly(ADP-ribose)polymerase (PARP), a protein that is involved in initiating DNA repair system, thus resulting in DNA damage. Mitochondrial respiration is also inhibited when exposed to HNO [10].

As NO and HNO have distinct physiological effects, with HNO being more cytotoxic than NO [3] and the ability of HNO to produce NO, with both as regulators of several mechanisms in normal levels and cytotoxic in high levels the importance of studying HNO effects becomes evident. The present study investigates the effect of NO and HNO under normal and low pO₂ on kidney mitochondria.
Material and methods

All chemicals used were from Sigma-Aldrich, Germany unless otherwise stated.

Male Sprague-Dawley rats were bought from Charles River, Germany and all experiments were approved by the local animal care and use committee at Uppsala University.

The buffer A solution used had the following composition in mmol/l: 250 sucrose, 10 HEPES, pH 7.4 and 300 mOsm/kg H2O.

The Liya 330 buffer was composed of: (in mmol/l: 70 sucrose, 220 mannitol, 5 MgCl2, 5 KPO4-, 10 HEPES, pH 7.4, 300 mOsm/kg H2O).

There exist different substrates which are known as NO donors, the one used in this experiment is diethylammonium(Z)-1-(N,N-diethylamino)diazen-1-ium-1, 2-diolate (DEA NONOate). DEA NONOate dissociated to free amine and NO in a pH-dependent manner following first order kinetics. The half-life of DEA NONOate is 2.16 minutes.

One important and known HNO donor is sodium trioxodinitrate (Na2N2O3) or Angeli’s salt (AS) (Switzer 2009), with a half-life of 2.3 min at physiological pH, decomposing according to first order kinetics.

Complex 1 substrate is Glutamate with final concentration of 10 mM.
Complex 2 substrate is Succinate and Rotenone with final concentration of 10 mM and 1mM. Complex 3 substrate is Duroquinol with final concentration of 100 µM.

Isolation of mitochondria:

Rats were decapitated, the kidneys excised and the renal capsule was removed. The kidneys were placed on ice in buffer A and the cortex and medulla were dissected. The renal (only cortex) tissue was homogenized in 10 ml ice-cooled buffer A solution in a potter-Elvehjem homogenizer rotating at approximately at 600 rpm. 1.5 ml of homogenate was transferred to eppendorf tube and centrifuged for 10 min, 4°C at 800 G. The supernatant from each tube was transferred to new tubes and centrifuged for 10 min, 4°C at 8000 G. The final pellets were washed with 300 µl of buffer A and kept on ice. Shortly before using each pellet for oxygen consumption measurements, each pellet was resuspended in 200 µl of buffer A.

Measurements of in vitro oxygen consumption:

Oxygen chambers with a Clark-type electrode (Oroboros instruments, Innsbruck, Austria) was calibrated with air-equilibrated buffer Liya 330.
To the chambers already containing 2000 µl of Liya 330 buffer, 20 µl of glutamate for complex I was added O2 consumption evaluated. After addition of mitochondria O2 consumption was noted after 5 minutes (denoted baseline). ADP-stimulated O2 consumption was measured by addition of 400 µM ADP and denoted ADP. After reaching a constant O2 consumption, 2 µl of DEA and AS was added to each chamber and O2 consumption was noted for each molecule after each t 1/2 (2.3 min for HNO and 2 min for NO) during 10-30 minutes. After each measurement, a sample from the O2 chamber was frozen for later analysis of protein concentration.

Same steps were repeated for other complexes using their respective substrates and for complex I under low oxygen levels with only DEA. Oxygen levels were decreased by injection of N2 in the gas phase and the chamber closed at the appropriate oxygen concentration.

Protein concentration measurement:

Protein concentration was determined with Bio-RAD DC protein assay using absorbance at 540 nm.
Results

Figure 6. Mitochondria oxygen consumption (in pmol O₂/s/mg protein) under complex I substrate (A, B), complex II substrate (C, D) and complex III substrate (E, F). The effects of NO and HNO are displayed in graphs A, C, E; NO and B, D, F; HNO.
No effects of NO were observed under any of the investigated substrates (Figure 6. A, C, E). Also HNO displayed no significant effect under studied substrates (Figure 6. B, D, F). Under low pO2 no effects of NO were observed (Figure 7).

**Figure 7.** Mitochondria oxygen consumption under low pO2 with effect of NO.

ADP stimulation and Respiratory control ratio (RCR) was significantly increased under low pO2 compared to normal pO2 (Figure 8, 9).

**Figure 8.** Mitochondria oxygen consumption under baseline and after ADP during normal and low pO2.
**Figure 9.** Respiratory control ratio (RCR) under normal and low pO$_2$. * denotes P< 0.05 vs. normal pO$_2$.

**Figure 10.** Example curve with HNO. Observe the large increase in oxygen consumption during approximately 20 minutes after addition of Angelis Salt, masking any effects that might be observed due to HNO.
Discussion

No effects of NO were seen although others have demonstrated effects of NO on oxygen consumption by inhibiting complex IV [9]. The reason why we could not demonstrate any effects of NO may be because of not having an optimal dose of NO or not a sufficient amount of mitochondria. However, NO have effects on mitochondria function and is associated with several diseases like diabetes and hypertension [5]. Thus, further experiments with higher levels of NO-donor are required.

No effects of HNO could be demonstrated. This is in contrast to Shiva 2004 since they could show inhibitory effects of HNO on complex II. The difference between the two studies is that, they have used an old apparatus for evaluating O$_2$ concentration which only can evaluate the slope, thus it does not show if O$_2$ consumption is completely linear. Meanwhile, the present study have used an Oroboros O$_2$K Oxygraph in which the O$_2$ concentration is converted to O$_2$ consumption and when the O$_2$-consumption line is horizontal then the decrease in O$_2$ concentration is linear. This enables the present study to more clearly evaluate HNO effects on mitochondria. As observed in this study, oxygen consumption was not linear and AS induced much noise in oxygen consumption measurements (see Figure 10). Stable value could be evaluated after 15-20 minutes, thus we might not see effects of HNO as HNO may have had effects during the first 15-20 minutes but a reversible effect and therefore it cannot be observed. Thus further studies such as in vivo studies are needed for studying the effects of HNO on kidney mitochondria.

Interestingly, respiratory control ratio (RCR) increased during low pO$_2$, meaning that mitochondria becomes more efficient, producing more ATP for the same amount of oxygen. This has not been previously demonstrated and warrants further studies. This might play a role in pathological states which are associated with a low tissue pO$_2$ such as diabetes and ischemic reperfusion injury (Figure 9).

Conclusion

No effects of HNO or NO could be demonstrated in kidney cortex mitochondria. HNO effects could not be evaluated properly due to methodological problems. The present study therefore highlights the importance of properly evaluating methods previously used.

Mitochondria under low oxygen tensions are display more efficient ATP-production compared to mitochondria from normal oxygen tension, an interesting find that warrants further studies.
References:


