Diabetes-induced Alterations in Renal Microcirculation and Metabolism

BY
FREDRIK PALM
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Abstract

Diabetes-induced renal complications, i.e. diabetes nephropathy, are a major cause of morbidity and mortality. The exact mechanism mediating the negative influence of hyperglycaemia on renal function is unclear, although several hypotheses have been postulated. Glucose-induced excessive formation of reactive oxygen species (ROS) and increased glucose flux through the polyol pathway are two major mechanisms that have recently gained increasing support. In order to investigate the development of hyperglycaemia-induced renal alterations further, it is of great importance to use an animal model in agreement with the pathological development in diabetic patients.

The aims of these investigations were to evaluate the streptozotocin (STZ)-diabetic Wistar Furth rat as a model for human diabetic nephropathy and to investigate involvement of ROS and the polyol pathway in development of diabetes-induced renal alterations.

The used STZ-diabetic animal model displayed several similarities with the progression of human disease, including initial hyperfiltration and albuminuria. However, the observed proteinuria could be partly linked to the STZ treatment per se, making the use of this animal model less suitable for research concerning diabetes-induced urinary protein leakage.

The diabetic state induced numerous alterations in renal function and metabolism, including increased oxygen consumption, decreased renal oxygen tension (pO₂), and altered lactate/pyruvate ratio. These renal alterations were preventable by daily treatment with either a radical scavenger (α-tocopherol) or an aldose reductase inhibitor (AL-1576).

In separate experiments the influence of nitric oxide (NO) on renal blood perfusion and pO₂ was investigated. The diabetic animals displayed a larger increase in renal NO activity after injecting the NO substrate L-arginine compared to non-diabetic animals, suggesting substrate limitation of the nitric oxide synthase during chronic hyperglycaemia.

In conclusion, the results from these investigations show that both ROS and the polyol pathway are involved in the development of diabetes-induced renal alterations in the STZ-diabetic Wistar Furth rat.

Keywords: diabetes mellitus, kidney, nephropathy, oxygen tension, haemodynamic, nitric oxide, oxygen consumption, radical oxygen species, polyol pathway

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"Äntligen en motgång"
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


Table of Contents

INTRODUCTION ......................................................................................................8
  Renal anatomy and physiology .................................................................8
  Renal architecture and blood perfusion ...................................................8
  Function of the kidneys .............................................................................9
  Renal metabolism .....................................................................................9
  Molecular mechanisms of hyperglycaemia-induced damage .................10
    Reactive oxygen species ........................................................................10
    Protein kinase C ....................................................................................10
    Advanced glycation end-products ......................................................11
    The polyol pathway ..............................................................................11
    The unifying mechanism .......................................................................12
  Animal models in diabetes research ......................................................12
AIMS OF THE INVESTIGATIONS........................................................................14
MATERIALS AND METHODS..............................................................................15
  General procedures applicable to all studies .........................................15
    Animals ..................................................................................................15
    Induction of diabetes ..............................................................................15
    Anaesthesia ...........................................................................................15
    Surgical preparation ..............................................................................15
    Estimation of glomerular filtration rate (GFR) ....................................16
    Urine analysis .........................................................................................16
    Statistical analysis ................................................................................16
  Specific procedures for each study ..........................................................16
    Islet isolation, culture and transplantation (Study II) ...........................16
    Metabolic cages (Study II) ...................................................................17
    Measurement of renal blood perfusion (Studies II-V) ..........................17
    Measurement of tissue oxygen tension (Studies II-V) ..........................18
    Measurement of tocopherol, TBARS and protein carbonyls (Study III) .19
    Isolation of renal cells (Studies III-IV) ..................................................20
    Measurement of in vitro oxygen consumption (Studies III-IV) ..........21
    Measurement of renal tissue pH (Study IV) ..........................................21
    In vivo measurement of NO-activity (Study V) ........................................22
Experimental protocols ..................................................................................23
  Study I .......................................................................................................23
  Study II .....................................................................................................24
  Study III ....................................................................................................24
  Study IV ....................................................................................................24
Study V ...........................................................................................................24

RESULTS AND COMMENTS ................................................................................25
Study I ..................................................................................................................25
Study II ................................................................................................................26
Study III ..............................................................................................................27
Study IV ..............................................................................................................29
Study V ..............................................................................................................31

DISCUSSION ........................................................................................................33
The streptozotocin-induced diabetic Wistar Furth rat ........................................33
Activation of molecular mechanisms ..................................................................33
  Reactive oxygen species .................................................................................34
  The polyol pathway .........................................................................................34
Mechanisms involved in the decreased renal tissue oxygen tension .................34
  Oxygen consumption ......................................................................................35
  Lactate and pH ...............................................................................................35
  Nitric oxide ....................................................................................................36
  Blood perfusion ..............................................................................................36

CONCLUSIONS ....................................................................................................37

ACKNOWLEDGMENTS .........................................................................................38

REFERENCES .......................................................................................................41
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-1576</td>
<td>spiro-(2,7-difluoro-9H-fluoren-9,4’-imidazolidine)2’,5’-dione</td>
</tr>
<tr>
<td>AR</td>
<td>Aldose reductase</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno sorbent assay</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pO₂</td>
<td>Oxygen tension</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SDH</td>
<td>Sorbitol dehydrogenase</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
</tbody>
</table>
INTRODUCTION

Diabetic patients are at high risk of developing renal dysfunction, and diabetes-induced renal complications are a major cause of morbidity and mortality. Common clinical signs of diabetic nephropathy are altered glomerular filtration rate (GFR) and subsequent increase in serum creatinine, albuminuria, proteinuria and end-stage renal failure. Morphological renal changes include basement membrane thickening, mesangial expansion and hypertrophy [1]. Patients with diabetes are also more susceptible to substances affecting the renal function, e.g. intravenous contrast media and non-steroid anti-inflammatory drugs [2, 3].

The Diabetes Control and Complication Trial Research Group showed that an important predictor of diabetes-induced complications is the degree of hyperglycaemia [4]. Even though increased extracellular glucose can alter the cellular function, the tissues most vulnerable to hyperglycaemia possess properties that also will increase the intracellular glucose. Interestingly, retinopathy, neuropathy and nephropathy all occur in tissues with insulin-independent glucose uptake. Within the renal structure there are two distinctive types of insulin-independent glucose transporters that are considered to be involved in the elevation of intracellular glucose concentration during hyperglycaemia. The sodium linked glucose transporters (Na+/glucose transporters) are located in the proximal tubular cells, and the glucose transporter-2 (GLUT-2) is present in various densities along the nephron [5].

Increased intracellular glucose concentrations cause activation of several biochemical pathways believed to be involved in the development of diabetic complications. Among these pathways is hyperglycaemia-induced increase in the formation of free radicals, i.e. increased oxidative stress, a hypothesis that has increasingly gained support during recent years [6, 7, 8].

Renal anatomy and physiology

Renal architecture and blood perfusion

The mammalian kidneys are composed of approximately one million functional units, nephrons, where filtration, reabsorption and excretion take place. There are two distinct types of nephrons, superficial and juxtamedullary (Fig. 1).
Approximately 20% of cardiac output is delivered to the kidneys, making the kidney one of the most highly perfused organs. The intrarenal distribution of blood flow is, however, highly heterogeneous, with only about 10% perfusing the deeper situated medullary structures. The blood perfusing the renal cortex passes through the glomeruli of the superficial nephrons, while the medullary blood flow is derived from the deeper situated glomeruli belonging to the juxtamedullary nephrons (Fig. 1).

Figure 1. The two major types of nephrons and the blood supply to the medullary region (a – superficial nephron, b – juxtamedullary nephron) Modified from [9].

After passing the glomeruli and the efferent arteriole of the juxtamedullary nephrons, the blood is transported in the descending vasa recta, which is in close contact with the ascending vasa recta. Close vicinity between these descending and ascending blood vessels is necessary in order to create a counter-current system that will retain the osmotic gradient between the cortex and the medulla.

Function of the kidneys
The kidneys play a pivotal role not only in the regulation of the fluid and electrolyte homeostasis, but also in the long-term regulation of arterial blood pressure and the excretion of metabolic waste products and water-soluble toxins. Numerous hormones are also produced within the different renal structures in order to maintain control of the internal milieu of the body. The kidney also produces significant amounts of alanine, serine and during certain conditions also glucose [10].

Renal metabolism
Even though the kidney only accounts for about 0.5% of the total body mass, it accounts for approximately 10% of the total oxygen consumption. The major consumer of energy within the renal structure is the basolaterally located Na+/K+-ATPase in the proximal tubular cells within the renal cortex. About 80% of the oxygen consumed in the mammalian kidneys is attributed to the reabsorption of electrolytes by the proximal tubular cells [11], while only 3 to 18% of the total oxygen consumption can be attributed to basal metabolism [10]. The metabolism within different parts of
the kidney is highly diverse and is likely to reflect the local energy demand and milieu in that specific region. The metabolism in the renal cortex has been found highly dependent on the availability of oxygen, i.e. aerobic metabolism. Glucose oxidation in the renal cortex is relatively low compared to that in the medulla, indicating a high glycolytic rate in the renal medulla. The high medullary glycolytic rate, together with the relatively low oxygen consumption, indicates the presence of anaerobic metabolism [10]. Notably, it has been shown that mitochondrial oxygen consumption rates are unaffected as long as the pO₂ is above 1 mmHg on the surface of the mitochondria.

Molecular mechanisms of hyperglycaemia-induced damage

Reactive oxygen species

Hyperglycaemia is closely associated with increased production of radicals, which has been linked to increased levels of NADH [6]. The cytosolic glucose is oxidized to pyruvate, which can either be converted to lactate or enter the mitochondrial tricarboxylic acid (TCA). NADH, generated either in the cytosol or by the TCA cycle, transfers electrons to NADH:ubiquinone oxidoreductase, complex I in the electron transfer chain. Further electron transfer includes complex III, IV and finally molecular oxygen. Electron transport through complex I, III and IV generates an electrochemical potential difference due to the created gradient of protons. A high potential difference stabilises superoxide-generating intermediates in the electron transport chain, resulting in increased formation of ROS [12].

There are several ways to prevent increased formation of hyperglycaemia-induced ROS in cell cultures. The use of inhibitors for complex I, complex II and uncoupler for oxidative phosphorylation that abolishes the mitochondrial membrane potential, have all been shown to prevent formation of ROS during hyperglycaemia [12]. Hyperglycaemia-induced formation of ROS activates at least three major pathways involved in the development of diabetic complications, namely activation of PKC, formation of AGEs and increased flux through the polyol pathway (Fig. 2) [12].

Protein kinase C

Already within days of the onset of hyperglycaemia there is an activation of PKC [13, 14]. Hyperglycaemia increases the de novo synthesis of diacylglycerol, an activator of PKC [14]. Activation of PKC has been shown to alter the activity of TGF-β and stimulate the synthesis of
Activation of protein kinase C (PKC)

Glucose-derived advanced glycation end-product (AGE)

Increased glucose flux through the polyol pathway

Hyperglycaemia

Reactive Oxygen Species (ROS)

Activation of protein kinase C (PKC)

Glucose-derived advanced glycation end-product (AGE)

Increased glucose flux through the polyol pathway

Hyperglycaemia

Diacylglycerol

PKC

MAPK ET-1 TGF-β ROS AGE NF-κB

ECM-Accumulation Apoptosis Proliferation

Hyperglycaemia + Proteins

Glucose

AR Sorbitol Fructose

Glucose-6-Phosphate

NADH

Glyceraldehyde-3-Phosphate

1,3-di-Phosphate Glycerate

Pyruvate Lactate

SDH HK

NADH/NAD+

NF-κB

Figure 2. Unifying mechanism for three major pathways involved in the development of hyperglycaemia-induced pathogenesis (AR – aldose reductase, HK – hexokinase, MAPK – mitogen-activated protein kinase, NF-κB – nuclear factor-kappa B, SDH – sorbitol dehydrogenase). Modified from [12, 16].

extracellular matrix [15]. Involvement of PKC in the activation of the mitogen-activated protein kinase (MAPK) pathway, which is an important regulator of cell growth, has also been reported [17]. The renal hypertrophy commonly seen in diabetic subjects is thought to be a result of MAPK pathway activation.

Advanced glycation end-products

Autooxidation of glucose to glycolaldehyde, decomposition of Amadori product to 3-deoxyglucosone, and conversion of glyceraldehyde-3-phosphate to methylglyoxal, produce reactive dicarbonyls, which can react with proteins to form AGEs [18]. Increased intracellular AGE formation will alter protein function, and alter the function of the extracellular matrix. AGE-modified plasma proteins will bind to AGE receptors inducing gene expression through nuclear factor-kappa B activation (NF-κB; Fig. 2) [19].

The polyol pathway

Increased intracellular glucose concentration has been shown to increase the flux through the
polyol pathway in vivo (Fig. 2), preferentially in tissues with insulin-independent glucose uptake. The enzyme aldose reductase reduces glucose to sorbitol, which is further oxidized to fructose by sorbitol dehydrogenase. These reactions are known as the polyol pathway and have been shown in both clinical and experimental studies to increase during manifest diabetes [16, 20]. The very high $K_m$ for aldose reductase (70 mmol/l) will result in an increased flux through the polyol pathway if the intracellular glucose concentration increases. Increased polyol pathway activity is known to alter the redox state, due to altered NADH/NAD$^+$ ratio [21]. Increased NADH/NAD$^+$ ratio will result in cellular abnormalities similar to those seen during hypoxia, despite the fact that the available oxygen is well above the hypoxic threshold. This condition is therefore referred to as pseudo-hypoxia [16]. The cellular alterations induced by increased polyol pathway activity are activation of pentose phosphate pathway, diacylglycerol and PKC [16]. Aldose reductase is located in the cytosol [22]. Within renal tissue, aldose reductase has predominantly been found in the medulla, while there seems to be little enzymatic activity in the renal cortex [23, 24]. Interestingly, NO has been found to possess inhibitory effects on aldose reductase due to modification of a cystein residue in the active region of the enzyme [25].

The unifying mechanism

Recently, Brownlee and co-workers have shown that the hyperglycaemia-induced formation of ROS by the electron transport chain, activates all of the major pathways involved in the development of diabetic complications (Fig. 2) [12]. Inhibition of the hyperglycaemia-induced excessive formation of ROS at the level of the mitochondrial membrane, resulted in complete lack of activation of PKC and the polyol pathway, and also prevented the formation of AGEs.

Animal models in diabetes research

A pivotal criterion when using animal models in any pathophysiological research is to have a close similarity between the pathological development in the animal model and the development in corresponding patients. A widely used animal model in diabetes research is the streptozotocin (STZ)-induced diabetes in rats. STZ [2-deoxy-2-(3-methylnitrosourea)-1-D-glucopyranose] is a broad spectrum antibiotic which is produced by *Streptomyces achromogenes*. Due to a molecular structure similar to glucose, the STZ molecule probably utilises the insulin-independent glucose transporter GLUT-2 to enter the cells [26]. The toxic effect of STZ is due to the highly reactive nitrosourea side chain,
which is likely to induce DNA strand break either as a result of the methylation of DNA bases or the direct formation of free radicals [27, 28]. STZ is a highly potent cytotoxin and is well known to be nephrotoxic [29]. In order to interpret the results from experiments using the STZ-induced diabetic rat model correctly, it is of great importance to know the influence of STZ *per se* on the investigated parameters.
AIMS OF THE INVESTIGATIONS

Study I  The aim of this study was to characterise the influence of diabetes on GFR in STZ-induced diabetic Wistar Furth rats from the early onset up to eight weeks after the induction of diabetes.

Study II  This study was designed to differentiate the effects of STZ per se from the long-term hyperglycaemia on renal function and metabolism in the STZ-induced diabetic Wistar Furth rat.

Study III This study was designed to investigate the effects of STZ-induced diabetes mellitus on regional renal blood perfusion, pO₂ and oxygen consumption. In addition, the role of ROS in the development of the observed renal alterations was investigated.

Study IV  This study investigated the role of the polyol pathway in the development of diabetes-induced changes in renal metabolism in relation to the previously reported altered renal pO₂.

Study V  The aim of this study was to investigate if an altered NO activity in the renal cortex in diabetic rats occurs, and if so, how this will effect cortical blood perfusion and pO₂.
MATERIALS AND METHODS

General procedures applicable to all studies

Animals
Approval was granted from the local animal ethics committee of Uppsala University for all the procedures in these studies. In all studies, inbred Wistar Furth rats from either B&K (Study I, II, III and V; Sollentuna, Sweden) or M&B (Study IV; Ry, Denmark) were used. All animals had free access to standard rat chow (R3, Ewos, Södertälje, Sweden) and water throughout the experimental periods. In study III, the chow was powdered and mixed with 5% (w/w) α-tocopherol (Merck Euro-lab, Stockholm, Sweden).

Induction of diabetes
Diabetes was induced by an intravenous injection of streptozotocin (STZ; 45 mg/kg BW; Sigma-Aldrich, St. Louis, Mo., USA) dissolved in water. The animals were considered diabetic if the blood glucose level was ≥ 15 mmol/l within 48 hours after the STZ-injection.

Anaesthesia
All rats were anaesthetized by an intraperitoneal injection of thiobutabarbital sodium (Inactin, 120 mg/kg BW for non-diabetic animals and 80 mg/kg BW for diabetic animals; Sigma-Aldrich).

Surgical preparation
Anesthetised animals were placed on a servo-regulated heating pad with a rectal probe, and a body temperature of 37°C was maintained throughout the surgical procedure. Catheters were placed in the femoral artery (-ies) and the femoral vein (-s) for blood pressure measurements, sampling, infusion of a Ringers solution and other substances. A subcostal flank incision was made to expose the left kidney, which was immobilised in a plastic cup and embedded in cotton wool soaked in saline. The renal surface was covered in paraffin oil to prevent evaporation. The urether was catheterized for urine collection, and the urine bladder was catheterized in order to allow drainage.
Estimation of glomerular filtration rate (GFR)

The GFR was estimated by determining the renal clearance of inulin. After surgery, the animals were allowed an equilibration period of 45 to 60 minutes followed by experimental periods where the clearance was determined. $^{3}$H-inulin (5 µCi/ml; American Radiolabeled Company, Mo., USA) dissolved in a Ringer solution was initially given as a bolus dose of 5 µCi and then infused (5 ml · kg $^{-1}$ · h$^{-1}$ for non-diabetic animals and 10 ml · kg · h$^{-1}$ for diabetic animals) via a catheter in the femoral vein. In blood and urine samples, the concentrations of $^{3}$H-inulin were determined by scintillation technique. The inulin clearance was calculated according to

$$\text{Clearance} = \frac{[\text{inulin}]_{\text{urine}}}{[\text{inulin}]_{\text{plasma}}} \times V_{\text{urine}}$$

where $V_{\text{urine}}$ denotes the urine flow rate in ml/min.

Urine analysis

Urine volumes were determined gravimetrically. The urinary concentrations of sodium and potassium were measured by flame spectrophotometry (IL543, Instrumentation Lab, Milan, Italy). The osmolality of the urine was determined by depression of the freezing point (Model 3MO, Advanced Instruments Inc, Needham Heights, Mass., USA).

Statistical analysis

All values are presented as means ± SEM. Comparisons between two different groups were performed using Student’s t-test for unpaired comparisons, whereas comparisons between two parameters within the same group were performed using Student’s t-test for paired comparisons. Multiple comparisons between different groups were performed using analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) test. Multiple comparisons within the same group were performed using repeated measures ANOVA followed by post hoc test for paired comparisons (Statview, Abacus Concepts, Berkeley, Calif., USA). For all comparisons, $p < 0.05$ was considered statistically significant.

Specific procedures for each study

Islet isolation, culture and transplantation (Study II)

Pancreatic islets were prepared by collagenase digestion (Boehringer-Mannheim, Mannheim, Germany) [30]. The islets were cultured free-floating for 4 to 7 days in RPMI 1640 medium supple-
mented with 10% vol/vol calf serum (Sigma-Aldrich) and the medium was changed every second day. After culture, approximately 1000 islets were packed in a butterfly needle (25 G) and thereafter slowly injected into the spleen of pentobarbital-anaesthetized (60 mg/kg BW intraperitoneal; Apoteket, Umeå, Sweden) syngeneic STZ-diabetic rats.

Metabolic cages (Study II)
The rats were individually placed in standard metabolic cages once a week in order to quantify the intake of food and water. The animals had unrestricted access to food and water. Before the start of the measurement, the animals were allowed a 24-hour period in the metabolic cages in order to minimize the influence of stress on the measurements. The urinary excretion of albumin and total protein were determined in urinary samples collected between 08:00 and 09:00 during the start of the experimental period. After the 24-hour experimental period, total urine production was quantified.

Total urinary protein excretion was determined by a Coomassie protein assay (Pierce, Rockford, Ill., USA), whereas urinary albumin was analyzed using a hypersensitive albumin ELISA with a rat albumin standard. Rat albumin standards and samples were coated in 96-well plates (Nunc, Roskilde, Denmark). After overnight incubation at 4°C, unbound surfaces were blocked with 1% (w/vol) caseine solution for 2 hours at 37°C. The wells were washed and incubated overnight at 4°C with a sheep anti-rat albumin antibody tagged with horseradish peroxidase (Biogenesis, Poole, UK). Tetramethylbenzidine (Sigma-Aldrich) was used as colorimetric substrate for the enzyme, and product accumulation was quantified at 370 nm.

Measurement of renal blood perfusion (Studies II-V)
Laser-Doppler flowmetry was used to estimate the regional renal blood flow in various parts of the renal tissue. The laser-Doppler probe (model 411, 0.45 mm o.d., PF 4001-2, Perimed, Stockholm, Sweden) was positioned in the renal structure using a micromanipulator and a stereomicroscope. The theory of the technique is to measure the velocity of red blood cells. This is achieved by sending out a light beam from a helium-neon laser and measuring the Doppler-shift of the reflected light. The Doppler-shift will be proportional to the number of red blood cells times the velocity of these cells relative to the light source. The usefulness of this technique in this kind of experiment has been thoroughly assessed elsewhere [31, 32, 33].
Measurement of tissue oxygen tension (Studies II-V)

Tissue pO₂ was measured amperometrically using Clark-type microelectrodes with a guard cathode (Fig. 3; Unisense, Aarhus, Denmark). The outer tip diameter of these electrodes was 3 to 5 µm. When polarizing the electrodes at –800 mV, relative to the reference electrode, the cathode reaction (reduction of oxygen) is thought to involve two steps. The first reaction is the reduction of dissolved oxygen [34]:

\[ \text{O}_2 + 2\text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2\text{O}_2 + 2\text{OH}^- \]

then

\[ \text{H}_2\text{O}_2 + 2e^- \rightarrow 2\text{OH}^- \]

The overall cathode reaction would then be:

\[ \text{O}_2 + 2\text{H}_2\text{O} + 4e^- \rightarrow 4\text{OH}^- \]

The most likely anode reaction is [34]:

\[ 4\text{Ag} + 4\text{Cl}^- \rightarrow 4e^- + 4\text{AgCl} \]

The H₂O₂ is a stable intermediate, but the kinetics will be more complicated if H₂O₂ is allowed to diffuse away or some other chemical causes decomposition back to O₂ and H₂O. This latter phenomenon was observed when

Figure 3. Schematic drawing of an oxygen microelectrode (modified from [35]).
measuring oxygen consumption from cells that had been pre-
treated with the radical scavenger 4-hydroxy-tempo. During these
measurements the electrodes be-
came unstable and finally unus-
able (unpublished observation).
In the in vivo experiments, the
oxygen electrodes were inserted
into the tissue using a microma-
nipulator and a stereomicroscope.
The obtained current when using
Clark-type electrodes in vivo was
~10 to 100 nA, which was re-
corded by picoamperemeters
(University of Aarhus, Aarhus,
Denmark). The position of the
electrodes was verified at the end
of each experiment when the kid-
ney was dissected.

Measurement of tocopherol,
TBARS and protein
carbonyls (Study III)
Renal tissue samples were thawed
and homogenized in ice-cold dis-
tilled water (0.2 g/ml). Tissue
concentrations of \( \alpha \)-tocopherol
were measured as previously de-
scribed [36, 37]. 500 µl of tissue
homogenate was mixed with 500
µl methanol (VWR International,
Stockholm, Sweden), 2 ml of
hexane was added and the sam-
ple was manually mixed for
three minutes and thereafter cen-
trifuged at 3000 rpm for 10 min-
utes. The hexane phase was col-
clected for further analysis. The
samples were separated with
HPLC using a Spherisorb amin-
column (4.6 x 250 mm; Phase
Separation, Deeside, UK). The
system was isocratic with isooc-
tane/tert-butyl-methyl-
ether/methanol (75/25/5) as mo-
bile phase at a flow of 1 ml/min.
The effluent was analysed
fluorometrically (Shimadzu RF-
10A, Shimadzu, Kyoto, Japan)
with an excitation wavelength of
295 nm and an emission wave-
length of 327 nm. The sample
values were compared to standard
values from \( \alpha \)-tocopherol (VWR
International).

Thiobarbituric acid reactive
substances (TBARS) were deter-
mined fluorometrically after boil-
ing the samples with thiobarbi-
turic acid. 200 µl of tissue ho-
mogenate was heated to 97°C for
60 minutes together with 250 µl
42 mmol/l thiobarbituric acid
(Merk, Darmstadt, Germany).
Standard samples were prepared
from malondialdehyde-bis-
diethylacetate (Merk-Schuchart,
Schuchart, Germany). The sam-
ple was subsequently cooled on
ice and precipitated with a mix-
ture of methanol and 1 mol/l
NaOH (91:9) and centrifuged at
3000 rpm for five minutes. Fluo-
rescence was then measured in the
supernatant; excitation wave-
length was 532 nm and emission
wavelength 553 nm, using Perkin-
Elmer LS 5B luminescence spec-
trophotometer (Perkin-
Elmer/Cetus, Norwalk, Conn.,
USA).
Renal concentrations of protein
carbonyls were determined using
a commercially available ELISA
kit (Zentech PC Test, Zenith
Technology, Dunedin, New Zealand). Homogenized samples (5 µl) were incubated for 45 minutes with a 2,4-dinitrophenylhydrazine solution yielding 2,4-dinitrophenylhydrazone groups on the protein carbonyls. The carbonylated proteins were then bound to a 96-well plate. Antibodies directed against the hydrazone groups were used in the ELISA. Serum albumin was used as standard with five different concentrations of hypochlorous acid-oxidized protein. Oxidation of O-phenylenediamine by horseradish peroxidase was used as chromotogenic reaction and the absorbance was measured at 490 nm using a spectrophotometer.

Isolation of renal cells (Studies III-IV)

The buffer solution had, if not stated otherwise, the following composition in mmol/l: 113.0 NaCl, 4.0 KCl, 27.2 NaHCO₃, 1.0 KH₂PO₄, 1.2 MgCl₂, 1.0 CaCl₂, 10.0 HEPES, 0.5 Ca-lactate, 2.0 glutamine. The buffer osmolality was adjusted to 298 ± 2 mOsm/kg H₂O as estimated with a freezing-point osmometer (Model 3MO), and the pH was adjusted to 7.40. Streptomycin (VWR International) was added to a final concentration of 50 U/ml. For non-diabetic rats, the buffer contained 5.8 mmol/l glucose, and for diabetic animals the buffer contained 23.2 mmol/l glucose (similar to the blood glucose concentrations in these latter animals). The renal cells were isolated as previously described [38, 39]. In brief, the rats were anaesthetized with thiobutabarbital, the kidneys were excised and the renal capsule was removed. The kidneys were placed on ice and the cortex and outer medulla were dissected under a stereomicroscope. Kidneys from two rats of the same group were pooled in order to increase the working material. The renal tissue was minced through a metallic mesh strainer and immediately placed in an ice-cooled buffer solution (cf. above) containing 0.05% (wt/vol) collagenase (Sigma-Aldrich). Thereafter, the minced tissue was incubated at 37°C for 90 minutes, while the buffer was equilibrated with 95% O₂/5% CO₂. At least once every five minutes, the buffer-tissue solution was manually mixed. After incubation, the cell suspension was cooled on ice and filtrated through graded filters with pore sizes of 180, 75, 53 and 38 µm, respectively. After filtration, the cells were pelleted by slow centrifugation (100 G, 4 minutes) and resuspended in a collagenase-free buffer. The rinsing procedure was repeated three times to ensure that no collagenase remained in the final cell suspension. The suspension was kept on ice until oxygen consumption was measured.
Measurement of \textit{in vitro} oxygen consumption (Studies III-IV)

Oxygen consumption was measured in a custom-made thermostatically controlled (37°C) gas-tight plexiglass chamber with a total volume of 1.100 ml [39]. The chamber was continuously stirred with an air driven magnetic stirrer. Modified Unisense 500 oxygen-sensing electrode was calibrated with air-equilibrated buffer solution as 228 µM O$_2$ and Na$_2$S$_2$O$_5$-saturated buffer as zero. After the calibration, 100 µl of cell suspension was injected into the chamber and the rate of oxygen disappearance was recorded. At the end of each experiment, a 100 µl sample was taken to determine the protein concentration using DC Protein Assay (Bio-Rad Laboratories, Hercules, Calif., USA). The oxygen consumption was calculated as the disappearance rate of oxygen adjusted for protein concentration.

Measurement of renal tissue pH (Study IV)

Tissue pH was measured in the renal tissue using pH microelectrodes (Fig 4; PH20, Unisense) connected to a pH meter (PHM240; Radiometer, Aarhus, Denmark).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4.jpg}
\caption{pH electrode specially manufactured for \textit{in vivo} purposes.}
\end{figure}

The pH electrodes follow Nernst equation, which simplified for this electrode is:

\[ E = -(0.05915) \log[H^+] + C \]

where \( E \) is the potential and \( C \) a constant depending on the reference electrode, salt bridge and internal electrolyte solution. The theoretically obtained potential per pH unit is 59.13 mV. Prior to \textit{in vivo} measurements, the electrode tips were precoated with 1% albumin (w/vol) dissolved in saline in order to correct for the influence of proteins on the obtained current (Fig. 5).

The electrodes were calibrated at 37°C before and after every experiment, using five standard buffers with pH 9.088±0.010, 7.386±0.010, 6.974±0.010, 6.841±0.010 and 4.020±0.010 (Radiometer).
In vivo measurement of NO-activity (Study V)

Nafion-coated Whalen-type microsensors were constructed as described by Buerk et al [41] and have a similar construction as the oxygen sensors first described by Whalen and co-workers 1967 [42]. Glass micropipettes with a tip diameter of 10 µm or less were filled with Woods metal. About 100 µm of the tip was left empty to allow gold to be plated on top of the Woods metal (Goodfellow, Huntingdon, UK). The gold-plated electrode was grinded to obtain a tip angle of about 45°. The Nafion membrane was applied by immersing the tip of the electrode in Nafion perfluorinated resin (5% wt dissolved in aliphatic alcohols; Sigma-Aldrich).

**Figure 5.** Calibration curve of a pH electrode and the influence of proteins on the obtained current. The obtained current/pH unit was 57.13 mV. After the electrode was coated with albumin, the obtained current was almost 1.5 mV lower per pH unit.

The sensitivity of these pH electrodes has previously been shown to be below 0.003 pH units, and the drift below 0.005 pH unit/h [40]. A stable reading was usually obtained within two minutes after insertion into the renal tissue.

**Figure 6.** Typical nitric oxide calibration curve for a Whalen-type microelectrode polarised at a constant voltage of +700 mV relative the Ag/AgCl reference electrode. A photo of the tip of a nitric oxide electrode (centre top) with a tip diameter < 10 µm.
The electrodes were polarized at a constant voltage of +700 mV relative to the Ag/AgCl-reference electrode. The electrochemical oxidation reaction for this electrode is:

\[
NO + 4OH^- \rightarrow NO_2^- + 2H_2O + 3e^-
\]

The obtained current was recorded by the hypersensitive battery powered Electrometer 602 Solid State (Keithley Instruments, Munich, Germany). Calibration was performed in deoxygenated buffer bubbled with either 100% nitrogen or a known mixture of NO and nitrogen [41]. From a calibration curve (Fig. 6) the ratio current per NO concentration was calculated and used when converting values from measurements to in vivo NO-activity. Due to the characteristics of the electrode and the unknown influence of other substances in vivo, only relative values of NO-activity can be obtained using this method. Substances influencing in vivo measurements of NO are preferentially ascorbic acid, catecholamines and high concentrations of electrolytes, but due to the recess in the tip of the electrode and the use of the highly selective Nafion membrane, this influence will be kept at an absolute minimum [41]. The alterations in NO-activity will also be much faster than any physiological process responsible for alterations in any of the other substances possessing the potential to influence the measurements of NO-activity.

The influence of different temperatures on the recorded current was evaluated. The magnitude of the current obtained for a specific change in NO concentration was independent of the temperature, but the current obtained for a specific absolute NO concentration was highly dependent on the temperature (Fig. 7).

**Experimental protocols**

**Study I**

Rats were divided into 9 groups: control and diabetic animals for 1 up to 8 weeks. At the time of the measurements, the animals were anaesthetized and after general surgery the GFR, the urine flow rate and the urinary excretion of osmolytes were measured during 30 to 60 minutes. At the end of each experiment the kidneys were
removed and the renal weight was measured.

Study II
Three groups were used in this study; control, STZ-treated diabetic and STZ-treated non-diabetic animals. The STZ-treated non-diabetic animals were transplanted with approximately 1000 pancreatic islets intrasplenically within 12 hours after the debut of hyperglycaemia, sufficient to reverse the STZ-induced hyperglycaemia. The animals were placed in metabolic cages weekly in order to determine the intake of food and water and the urinary excretion of osmolytes, albumin and total protein. The blood glucose and body weight were also monitored weekly. After four weeks the animals were anaesthetized and following general surgery the GFR, the urine flow rate and the excretion of osmolytes, albumin and total protein were determined. Thereafter the local renal blood flow and tissue pO₂ were measured.

Study III
The animals were divided into four experimental groups; non-diabetic and diabetic animals fed either R3 pellets or DL-α-tocopherol supplemented to the powdered pellets. Animals from each of the four groups were subjected to measurements of either regional renal blood flow and pO₂ or in vitro oxygen consumption. Renal tissue from the animals was frozen in liquid nitrogen for later analysis of α-tocopherol, lipid peroxidation and carbonylated proteins.

Study IV
Four groups were used; non-diabetic and diabetic animals with and without treatment with the aldose reductase inhibitor AL-1576.
Four weeks after the induction of diabetes/start of treatment, the animals were anaesthetized and subjected to measurements of regional renal blood flow and tissue pO₂, in vitro oxygen consumption, renal metabolites by microdialysis or local tissue pH.
The GFR and urinary excretion of electrolytes were also determined in the anaesthetized animals.

Study V
Anaesthetized non-diabetic and diabetic animals were subjected to simultaneous measurements of renal cortical NO-activity, pO₂ and blood perfusion before and after intravenous injections of the NO substrate L-arginine, followed by similar injection of the unspecific NOS inhibitor L-NAME. GFR, excretion of sodium, potassium and osmolytes were also measured during the entire experimental period.
RESULTS AND COMMENTS

Study I

Transient glomerular hyperfiltration in the streptozotocin-diabetic Wistar Furth rat

This study characterized the influence of STZ-induced diabetes mellitus on the GFR. The study revealed two important characteristics of the STZ-induced diabetic Wistar Furth rat model. Firstly, during week 1 to 5 following the injection of STZ, the GFR was markedly elevated compared to non-diabetic control animals (Fig. 8). After the fifth week, the GFR returned to the same filtration rates as in the non-diabetic animals. Secondly, the renal hypertrophy was evident already one week after the induction of hyperglycaemia.

Figure 8. The glomerular filtration rate (GFR) in non-diabetic control (grey horizontal bar) and streptozotocin-induced diabetic Wistar Furth rats (filled circles) 1 to 8 weeks after the induction of diabetes. Values are presented as mean±SEM. a denotes p<0.05 versus control group.
Study II

Differentiating between effects of streptozotocin *per se* and subsequent hyperglycemia on renal function and metabolism in the streptozotocin-diabetic rat model

The major findings of this study were that the proteinuria seen in these animals after the injection of STZ is due to the toxic properties of STZ *per se*, whereas the albuminuria is due to the subsequent hyperglycaemic state (Fig. 9). Alterations in other investigated parameters, such as renal weight, GFR, regional renal blood flow and renal tissue pO2, were all linked to the long-term diabetic state.

The transplantation of the pancreatic islets resulted in an instant reversal of the increased blood glucose in all transplanted animals. The serum insulin concentration in these animals was found to be similar to that of the non-diabetic control animals.

![Figure 9](image_url)

*Figure 9. The urinary excretion of protein (left) and albumin (right) in non-diabetic streptozotocin-induced diabetic (triangles) and cured STZ-injected (circles) Wistar Furth rats. The grey horizontal lines denote protein and albumin excretion respectively, for non-diabetic control animals. Values are presented as mean±SEM. a denotes p<0.05 versus start of the experiments within the same group and b denotes p<0.05 versus diabetic animals.*
Study III

Reactive oxygen species cause diabetes-induced decrease in renal oxygen tension

The results of this study showed that diabetic animals have a decreased renal tissue $\text{pO}_2$ at every corresponding depth, but preferentially in the renal medulla, compared to non-diabetic control animals (Fig. 10). Treatment with the radical scavenger $\alpha$-tocopherol (vitamin E) throughout the course of diabetes prevented the decrease in $\text{pO}_2$, indicating ROS to be a crucial mechanism.

**Figure 10.** Tissue oxygen tension at different depth from the renal surface in non-diabetic control (filled circles), non-diabetic animals fed vitamin E (empty circles), diabetic animals (filled triangles) and diabetic animals fed vitamin E (empty triangles). a denotes $p<0.05$ versus non-diabetic control animals, b denotes $p<0.05$ non-diabetic fed vitamin E versus diabetic animals fed vitamin E and c denotes $p<0.05$ diabetic animals fed vitamin E versus non-diabetic animals. Values are presented as mean±SEM (CMB – anatomical border between cortex and medulla, OM/IM – anatomical border between outer and inner medulla).
The oxygen consumption was markedly increased in both cortical and medullary cells from diabetic animals. Treatment with α-tocopherol prevented this increase (Table 1).

The diabetic state resulted in elevated lipid peroxidation and protein carboxylation, preferentially within the renal medulla (Table 2).

**Table 1. Oxygen consumption in renal cortical and medullary cells from non-diabetic and diabetic rats with and without treatment with α-tocopherol.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cortical cells</th>
<th>Medullary cells</th>
<th>Cortical cells + 1mmol/1 ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15/8</td>
<td>29.6±2.7</td>
<td>25.6±2.8</td>
<td>19.6±2.0</td>
</tr>
<tr>
<td>Control+tocopherol</td>
<td>16/9</td>
<td>27.7±1.7</td>
<td>30.4±3.9</td>
<td>19.6±1.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>13/7</td>
<td>41.3±2.8</td>
<td>55.7±8.2</td>
<td>24.6±2.3</td>
</tr>
<tr>
<td>Diabetic+tocopherol</td>
<td>16/15</td>
<td>24.7±1.6</td>
<td>23.1±3.4</td>
<td>18.0±2.5</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM. a denotes p<0.05 versus non-diabetic control group, b denotes p<0.05 versus corresponding untreated animals and c denotes p<0.05 versus corresponding non-ouabain treated cells.

**Table 2. Concentrations of α-tocopherol, thiobarbituric acid reactive substances (TBARS) and carbonylated protein in different tissues from non-diabetic control and diabetic animals with or without treatment with α-tocopherol.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + tocopherol</th>
<th>Diabetic</th>
<th>Diabetic + tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tocopherol renal cortex (µg/g)</td>
<td>0.28±0.07</td>
<td>1.49±0.14</td>
<td>0.11±0.02</td>
<td>1.65±0.17</td>
</tr>
<tr>
<td>α-tocopherol renal medulla (µg/g)</td>
<td>67.6±10.7</td>
<td>140.0±12.6</td>
<td>43.5±4.7</td>
<td>217.7±14.7</td>
</tr>
<tr>
<td>α-tocopherol liver (µg/g)</td>
<td>16.3±2.8</td>
<td>176.5±11.7</td>
<td>35.7±3.3</td>
<td>407.5±77.6</td>
</tr>
<tr>
<td>TBARS cortex (nmol/g)</td>
<td>43.7±1.9</td>
<td>25.2±1.8</td>
<td>45.1±4.2</td>
<td>30.6±0.9</td>
</tr>
<tr>
<td>TBARS medulla (nmol/g)</td>
<td>30.5±2.6</td>
<td>38.2±15.4</td>
<td>159.4±36.6</td>
<td>80.4±17.8</td>
</tr>
<tr>
<td>Protein carbonylation medulla (nmol/mg protein)</td>
<td>1.24±0.20</td>
<td>1.15±0.09</td>
<td>1.73±0.07</td>
<td>1.01±0.10</td>
</tr>
</tbody>
</table>

All values are means±SEM. a denotes p<0.05 when compared to control group, b denotes p<0.05 when compared to α-tocopherol treated non-diabetic animals and c p<0.05 when compared to corresponding untreated diabetic animals.
Study IV
Polyol pathway-dependent disturbances in renal medullary metabolism in experimental insulin-deficient diabetes mellitus in rats

This study resulted in four major findings. Firstly, the diabetes-induced decrease in renal pO$_2$ previously reported in paper III is mediated by the polyol pathway, due to the fact that inhibition of the enzyme aldose reductase prevented this decrease (Fig. 11). Secondly, the hyperglycaemic state resulted in a markedly increased medullary lactate/pyruvate ratio despite normal levels of purine base metabolites, indicating pseudohypoxia in this region (Fig. 12). This was further supported by the complete reversal of the increased lactate/pyruvate ratio when inhibiting the enzymatic activity of aldose reductase.

Figure 11. The tissue oxygen tension in control animals (filled circles), control animals treated with an aldose reductase inhibitor (empty circles), diabetic animals (filled triangles) and diabetic animals treated with an aldose reductase inhibitor (empty triangles). a denotes p<0.05 versus control animals, b denotes p<0.05 versus diabetic animals and c denotes p<0.05 versus treated control animals. Values are presented as mean±SEM (CMB – anatomical border between cortex and medulla, OM/IM – anatomical border between outer and inner medulla).
Thirdly, the increased lactate/pyruvate ratio resulted in a decreased tissue pH in the renal medulla (Fig 13). The altered pH was also preventable by inhibition of aldose reductase.

**Figure 12.** The lactate/pyruvate ratio in the renal cortex (left) and medulla (right) in control, diabetic and AL-1576 treated rats. a denotes p<0.05 versus non-diabetic control animals, whereas b denotes p<0.05 versus diabetic animals. Values are presented as mean±SEM.

**Figure 13.** Renal tissue pH in control (circles) and diabetic animals (triangles) with and without treatment of an aldose reductase inhibitor (empty and filled symbols, respectively). a denotes p<0.05 versus corresponding cortex value within the same group, whereas b denotes p<0.05 versus control animals within the same region. Values are presented as mean±SEM.
Fourthly, the increased cellular oxygen consumption seen in diabetic animals in both the renal cortex and the medulla was preventable by inhibition of aldose reductase (Table 3).

**Table 3.** Oxygen consumption in renal cortical and medullary cells from control and diabetic rats with or without treatment with an aldose reductase inhibitor (AL-1576) throughout the duration of diabetes.

<table>
<thead>
<tr>
<th></th>
<th>O2 consumption (nmol · mg protein⁻¹ · min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortical cells</td>
</tr>
<tr>
<td></td>
<td>(n=7-10)</td>
</tr>
<tr>
<td>Control</td>
<td>33.2±2.4</td>
</tr>
<tr>
<td>Control + AL-1576</td>
<td>25.6±1.6°</td>
</tr>
<tr>
<td>Diabetic</td>
<td>40.0±2.9</td>
</tr>
<tr>
<td>Diabetic + AL-1576</td>
<td>33.2±3.6</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM. ° denotes p<0.05 versus control group, b denotes p<0.05 versus corresponding untreated diabetic animals, c denotes p<0.05 versus corresponding non-ouabain treated cells and d denotes p<0.05 versus corresponding cortical cells within the same group.

**Study V**

**The effect of nitric oxide on renal microcirculation, oxygenation and function in streptozotocin-induced diabetes mellitus**

The major finding of this study was that NO activity in diabetic animals after both injection of L-arginine and L-NAME was markedly different compared to non-diabetic control animals (Fig. 14). The hemodynamic response in the diabetic animals after injection of L-arginine and L-NAME was also different compared to control animals (Fig. 15).
Figure 14. Nitric oxide activity in the renal cortex of control and diabetic animals. a denotes p<0.05 versus corresponding control group. Values are presented as mean±SEM.

As previously reported in both paper III and IV, the baseline tissue pO2 in the renal cortex of the diabetic animals was significantly lower as compared to non-diabetic animals (Fig. 15). Alterations of the NOS activity by the injections of L-arginine and L-NAME altered the pO2 in the diabetic animals differently compared to the control animals.

Figure 15. Average cortical blood perfusion (left) and oxygen tension (right) before and after injection of L-arginine and L-NAME in control and diabetic animals. a denotes p<0.05 versus control group at corresponding time and b denotes p<0.05 versus baseline within the same group. Values are presented as mean±SEM.
DISCUSSION

Hyperglycaemia either induces or seriously alters several cellular events that, if left unattended, will result in severe tissue damage and organ dysfunctions. The present work evaluates the most commonly used animal model in diabetes research. The model reveals several hyperglycaemia-induced alterations in renal metabolism and function.

The streptozotocin-induced diabetic Wistar Furth rat

In paper I and II, the STZ-diabetic Wistar Furth rat model was evaluated with regard to renal alterations. Paper I characterized the development of GFR alteration after the induction of diabetes. Similar to the development in the diabetic patients, the diabetic rats had a pronounced and transient glomerular hyperfiltration during the initial phase after the onset of diabetes.

STZ is known to be nephrotoxic and in paper II the renal effect of STZ per se was isolated and investigated. This was possible due to a new approach, where animals injected with STZ were transplanted with a sufficient number of pancreatic islets in order to reverse the increased blood glucose, immediately after the onset of hyperglycaemia. The results showed that the proteinuria was mainly due to the toxic effects of STZ, while alterations in GFR, urinary excretion of albumin, renal blood perfusion and oxygen tension were due to the subsequent hyperglycaemia. The urinary leakage of albumin is generally considered to be a result of glomerular damage [43], while the urinary leakage of other proteins is viewed as preferentially being due to tubular damage [44]. The transport of STZ across the cell membranes has been shown to be highly specific and mediated by specific glucose transporters [26]. The molecular structure of STZ is very similar to the glucose molecule. The insulin-independent glucose uptake in the proximal tubular cells by the sodium linked glucose transporter (Na+/glucose transporter) is one potential mechanism accounting for the tubulotoxic properties of the STZ.

Activation of molecular mechanisms

Paper III and IV investigated the role of ROS and the polyol pathway in the development of renal alterations in the STZ-induced diabetic animal model.
Reactive oxygen species

The formation of ROS, measured as lipid peroxidation and protein carboxylation, was increased in the renal medulla of the diabetic rats (paper III). At present, the consequences of lipid and protein damages on renal function remain to be determined. The increased ROS formation also caused increased renal oxygen consumption and decreased renal pO\(_2\). Daily treatments with the free radical scavenger \(\alpha\)-tocopherol resulted in increased renal tissue concentrations of this vitamin, and almost complete prevention of the observed hyperglycaemia-induced renal alterations.

A probable mechanism accounting for the elevated ROS levels in the diabetic rats is an increased formation of radicals by the superoxide-generating intermediates in the electron transport chain, due to an increased proton gradient [12]. Excessive amount of ROS has been shown to activate several mechanisms responsible for the development of diabetes complications, including formation of AGEs, increased flux through the polyol pathway and increased degradation of NO [8, 12, 45].

The polyol pathway

Increased lactate/pyruvate ratios were found in both the renal cortex and the renal medulla of diabetic animals, even though the oxygen concentrations were well above the level of hypoxia. Increased flux through the polyol pathway has been shown to alter the redox state of the tissue [16, 21]. This state is characterized by an increased lactate/pyruvate ratio due to excessive supply of NADH, that will increase the enzymatic activity of lactate dehydrogenase, resulting in a higher lactate/pyruvate ratio. This will occur even if the oxygen supply is sufficient for unlimited mitochondrial respiration, and has therefore been named pseudohypoxia.

The altered redox state activates an intracellular cascade of events that will eventually cause both morphological and physiological changes. [14, 46, 47]. Among the known mechanisms activated by increased flux through the polyol pathway are increased formation of diacylglycerol, activation of PKC and increased production of radicals [16].

Mechanisms involved in the decreased renal tissue oxygen tension

The in vivo pO\(_2\) in any tissue of an animal is the result of the net delivery of oxygen and the oxygen consumption within that tissue. Altering any of these two parameters will undoubtedly affect the pO\(_2\) in that specific tissue. The results from paper III and IV show that the diabetic state induces decreased renal tissue pO\(_2\) throughout the renal structure. In
paper V the in vivo NO activity was modulated while the effects on tissue pO2 and blood perfusion were recorded.

**Oxygen consumption**

The renal oxygen consumption was found to be increased in the cells isolated from diabetic animals (paper III and IV). This finding is in conjunction with previous reports [38, 48] and is linked to upregulation of Na\(^+\)/K\(^+\)-ATPase, since the ouabain sensitive oxygen consumption was significantly increased. Notably, the oxygen consumption was increased in diabetic animals independently of changes in GFR. This suggests that other mechanisms apart from an altered tubular sodium load are involved in the increased oxygen consumption. Increased flux through the Na\(^+\)/glucose transporter, as a result of excessive tubular load of glucose due to the increased blood glucose concentration, has been shown to increase the Na\(^+\)/K\(^+\)-ATP-ase activity [38, 48]. Another possible mechanism is a decreased inhibition of the oxygen consumption by NO (cf. below). However, the hyperglycaemic state is known to induce enhanced expression of uncoupling protein-2, increase gluconeogenesis and increase fatty acid metabolism, all resulting in increased oxygen consumption [48, 49, 50]. During normoglycaemic conditions there are no insulin independent Na\(^+\)/glucose transporters in the renal medulla, but the low-affinity GLUT-2 has been found in significant amounts all along the nephron [5]. A challenging speculation is that the increased medullary tubular glucose load, found in the diabetic animals, can induce protein expression of the Na\(^+\)/glucose transporter, resulting not only in increased intracellular glucose concentrations, but also in increased cellular energy demand and subsequent oxygen consumption.

**Lactate and pH**

The medullary blood perfusion is derived through the closely located descending and ascending vasa recta (Fig. 1). This arrangement results in a counter-current mechanism, necessary in order to keep up the osmotic gradient between the renal cortex and the medulla. Notably, even during normal physiological conditions, the oxygen is shunted in the opposite direction, resulting in low delivery of oxygen to the medullary structures. As shown in paper IV, the hyperglycaemic state induces a pronounced increase in the lactate/pyruvate ratio, predominantly due to increased lactate production. This increased lactate concentration will decrease the pH and the protons will recirculate, in the same manner as electrolytes, in the medullary structures due to the counter current mechanism in the vasa recta. When acidic blood from the medullary structures
comes in the vicinity of the arterial blood in the descending *vasa recta*, the shunting of oxygen (from descending to ascending vessels) will increase as a result of the effect of the protons on the dissociation of oxygen from the haemoglobin. The net result will be an even further reduced oxygen delivery to the renal medulla, despite the fact that the blood perfusion is unaffected.

Nitric oxide

The bioavailability of NO is of great importance not only for the hemodynamic regulation, but also for the regulation of oxygen consumption within many tissues [45]. Koivisto and co-workers showed that NO inhibits the mitochondrial respiration in the proximal tubular cells, and that the inhibition is proportional to the inverse square of the oxygen concentration [39, 51]. During normal physiological conditions, the influence of NO inhibition is likely to be significant in the renal medulla due to the normally low tissue pO2. The results from paper V clearly demonstrated that the bioavailability of NO in the STZ-diabetic animals was markedly lower compared to non-diabetic animals. The reason for the decreased NO activity was probably a limitation in the available NO substrate L-arginine, since addition of L-arginine caused a more pronounced NO increase in the diabetic animals. This might influence the mitochondrial oxygen consumption rate and, thus, the renal tissue pO2.

Blood perfusion

The reported effects of hyperglycaemia on the renal blood perfusion in the STZ-induced diabetes animal model are highly diverse [52]. In the present studies, the basal regional renal blood perfusion was unchanged by the diabetic state. The contribution of an altered renal blood perfusion to the observed decrease in renal pO2 is therefore likely to be of minor importance.
CONCLUSIONS

Viewed as a whole, the results from the first two studies show that the STZ-induced diabetic Wistar Furth rat model may be a suitable animal model for studies of the mechanisms involved in the development of diabetic nephropathy. Paper III-V reveals a pronounced effect of long-term hyperglycaemia on the renal metabolism and regulation of blood perfusion. The results also support the conclusions that:

- STZ-induced diabetes in Wistar Furth rats results in a transient increase in GFR during the first 4 to 5 weeks after induction of diabetes.

- In the STZ-induced diabetic rat model, the total proteinuria is at least partly due to STZ toxicity *per se*, while alterations in urinary albumin excretion, GFR, renal weight and tissue pO₂ are due to long-term hyperglycaemia.

- Oxidative stress occurs in renal tissue of STZ-induced diabetic rats predominantly in the medullary region, and is related to augmented oxygen consumption and, consequently, impaired pO₂.

- Renal alterations induced by increased oxidative stress can be prevented by continuous treatment with the radical scavenger α-tocopherol.

- Long-term hyperglycaemia induces increased lactate/pyruvate ratio (pseudohypoxia) and decreased tissue pH in the renal medulla.

- The diabetes-induced alterations in medullary tissue pO₂, oxygen metabolism and tissue pH are mediated through the polyol pathway and can be prevented by inhibition of the enzymatic activity of aldose reductase.

- STZ-induced diabetes mellitus results in impaired NO activity, probably due to limitation in the availability of the NO substrate L-arginine.

In conclusion, the results from these investigations show that both ROS and the polyol pathway are involved in the development of diabetes-induced renal alterations in the STZ-diabetic Wistar Furth rat.
ACKNOWLEDGMENTS

This thesis is the result of the collaboration with a number of people. I would especially like to thank,

The man, the myth, the supervisor Per Liss for teaching me the art of “äntligen en motgång”. He once said: “There was a time when you played All along the watchtower by Bob Dylan on the guitar and looked the babes deep in the eyes and longed for ……a beer. One gets older and by now you know what to give priority to”.

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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)