Reduced Nitric Oxide Concentration in the Renal Cortex of Streptozotocin-Induced Diabetic Rats
Effects on Renal Oxygenation and Microcirculation

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Nitric oxide (NO) regulates vascular tone and mitochondrial respiration. We investigated the hypothesis that there is reduced NO concentration in the renal cortex of diabetic rats that mediates reduced renal cortical blood perfusion and oxygen tension (PO2). Streptozotocin-induced diabetic and control rats were injected with L-arginine followed by NO-nitro-L-arginine-methyl-ester (L-NAME). NO and PO2 were measured using microsensors, and local blood flow was recorded by laser-Doppler flowmetry. Plasma arginine and asymmetric dimethylarginine (ADMA) were analyzed by high-performance liquid chromatography. L-Arginine increased cortical NO concentrations more in diabetic animals, whereas changes in blood flow were similar. Cortical PO2 was unaffected by L-arginine in both groups. L-NAME decreased NO in control animals by 87 ± 15 nmol/l compared with 45 ± 7 nmol/l in diabetic animals. L-NAME decreased blood perfusion more in diabetic animals, but it only affected PO2 in control animals. Plasma arginine was significantly lower in diabetic animals (79.7 ± 6.7 vs. 127.9 ± 3.9 nmol/l), whereas ADMA was unchanged. A larger increase in renal cortical NO concentration after L-arginine injection, a smaller decrease in NO after L-NAME, and reduced plasma arginine suggest substrate limitation for NO formation in the renal cortex of diabetic animals. This demonstrates a new mechanism for diabetes-induced alteration in renal oxygen metabolism and local blood flow regulation. Diabetes 54:3282–3287, 2005

RESEARCH DESIGN AND METHODS

Diabetes induction. Diabetes was induced by an injection of streptozotocin (STZ; 45 mg/kg body wt; Sigma-Aldrich, St. Louis, MO) in the tail vein. Animals were considered diabetic if blood glucose concentrations increased to ≥15 mmol/l within 24 h after STZ injection and remained elevated. Blood glucose concentrations were determined with test reagent strips (Medibence, Bedford, MA) from blood samples obtained from the cut tip of the tail in all animals.

Surgical procedures. At 4 weeks after allocation to the study, all animals were anesthetized with an intraperitoneal injection of thiobutabarbital (120 mg/kg body wt nondiabetic, 80 mg/kg body wt diabetic animals, Inactin; Sigma-Aldrich), placed on an operating table maintained at 37°C, and tracheostomized. Polyethylene catheters were placed in the right femoral artery and in the right femoral vein. The arterial catheter was used to monitor blood pressure (Statham P23DB, Statham Laboratories, Los Angeles, CA). The catheter in the vein was used for infusion of Ringer solution to compensate for loss of body fluid and for infusion of substances. The urinary bladder was catheterized to allow urinary drainage. The left kidney was exposed by a left subcostal flank incision, immobilized in a plastic cup, and embedded in pieces competitive inhibitor of oxygen consumption (5) at the level of cytochrome oxidase, the terminal electron acceptor in mitochondria (6). Therefore, the magnitude of the inhibition of oxygen consumption by NO will increase at low PO2 (7).

Long-term hyperglycemia is associated with increased oxidative stress, i.e., increased production of reactive oxygen species (ROS) (8–11). ROS can react with NO, forming peroxynitrite, and thus decrease the bioavailability of NO (11). The bioavailability of NO and formation of peroxynitrite are also highly dependent on superoxide dismutase, as modeled by Buerk et al. (12). Decreased influence of NO has therefore been suggested to be involved in the increased renal cortical cellular oxygen consumption closely associated with manifest diabetes (10,13,14). Furthermore, involvement of endogenous competitive NOS inhibitor asymmetric dimethylarginine (ADMA) in the development of vascular complications has gained increasing support over the last few decades (15).

Because NO regulates the delivery of oxygen to tissue both by setting the level of vascular tone and blood pressure and by inhibiting cellular oxygen consumption, alterations in NO activity might contribute to the development of diabetes-induced renal hypoxia. The current study aimed to investigate whether there is a diabetes-induced alteration in the NO concentration in the renal cortex and, if so, study the importance of this for renal cortical blood perfusion and oxygenation.

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ADMA, asymmetric dimethylarginine; GFR, glomerular filtration rate; L-NAME, N-nitro-L-arginine-methyl-ester; NOS, NO synthase; ROS, reactive oxygen species; SDMA, symmetric dimethylarginine; STZ, streptozotocin.

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of cotton wool soaked in saline. The surface of the kidney was covered with paraffin oil (Apoteksbolaget, Gothenburg, Sweden) to avoid evaporation. The left ureter was catheterized for collection of urine for subsequent analysis.

### Simultaneous measurements of renal NO activity, blood perfusion, and oxygen tension

Animals were allowed a 60-min recovery period followed by 3×20 min of measurements. After 20 min of control measurements, a single bolus dose of the NO substrate L-arginine (50 mg/kg body wt i.v.; Sigma-Aldrich). After another 20 min, the unspecific NOS inhibitor Nω-nitro-L-arginine-methyl-ester (L-NAME; Sigma-Aldrich; 10 mg/kg body wt i.v.). Glomerular filtration rate (GFR) was estimated by measurements of inulin clearance. For this purpose, [3H]inulin (185 kBq/ml; American Radiolabeled Company, St. Louis, MO) was dissolved in saline was initially given as a bolus dose of 185 kBq and then infused (5 ml·kg⁻¹·h⁻¹ i.v.). Urine and arterial blood samples were taken for subsequent analyses. Renal cortical NO concentration was measured by recessed Whalen-type gold microsensors (tip dimensions ≤5 μm), which were fabricated from glass micropipettes (16). Microsensors were polarized at +700 mV (amperometric method) relative to an Ag/AgCl reference electrode. NO microsensors were calibrated in deoxygenated buffer, bubbled with either 100% nitrogen or a mixture of NO and nitrogen (17). Because of the characteristics of these microsensors and the fact that calibration only can be achieved in vitro, merely relative changes of NO activity can be recorded during measurements in vivo. Cortical blood perfusion was measured with laser-Doppler flowmetry (probe 411, 0.45 mm optical density; PF 4001–2, Perimed, Stockholm, Sweden). Oxygen tension (PO2) was measured with a polarographic technique, using modified Clark-type microelectrodes (4–6 μm outer diameter; Unisense, Aarhus, Denmark) (18,19). Electrodes were two-point calibrated in water saturated with Na2S2O5 to avoid evaporation. The surface of the kidney was covered with cotton wool soaked in saline. The surface of the kidney was covered with paraffin oil (Apoteksbolaget, Gothenburg, Sweden) to avoid evaporation. The left ureter was catheterized for collection of urine for subsequent analysis.

### Measurements of blood perfusion–dependent renal oxygenation

A clamp was placed on the left renal artery. Renal cortical blood perfusion and PO2 were measured, as described above, after renal artery occlusion in nondiabetic (n = 7) and diabetic animals (n = 7). The clamp was tightened stepwise 5–6 times in each animal. Readings of blood perfusion and PO2 were performed after each time the clamp was tightened and stable values had been observed for at least 1 min.

#### Measurements of blood perfusion

Renal cortical blood perfusion and PO2 were measured, as described above, after renal artery occlusion in nondiabetic (n = 7) and diabetic animals (n = 7). The clamp was tightened stepwise 5–6 times in each animal. Readings of blood perfusion and PO2 were performed after each time the clamp was tightened and stable values had been observed for at least 1 min.

#### Measurements of urine parameters

The radioactivity of [3H]inulin in plasma (10 μl) and urine (1 μl) was measured by liquid scintillation. GFR was then calculated as the clearance of [3H]inulin. Urine volumes were measured gravimetrically, osmolality by use of a freezing point technique (model 3MO; Advanced Instruments, Norwood, MA), and urinary sodium and potassium concentrations by use of a flame spectrophotometer (IL545; Instrumentation Lab, Milan, Italy).

#### Results

Blood glucose concentrations were 5.7 ± 0.2 mmol/l in control animals (n = 22) and 23.2 ± 0.5 mmol/l (P < 0.05 vs. control) in 4-week diabetic animals (n = 25). Renal weights were increased in 4-week diabetic animals compared with control animals (1.38 ± 0.05 g, n = 25, and 0.97 ± 0.02 g, n = 22, respectively; P < 0.05).

Injection of L-arginine caused a larger increase in renal cortical NO concentration in diabetic animals than in control animals (Fig. 1). Administration of L-NAME resulted in a pronounced decrease in renal cortical NO concentration in both groups, but with the largest decrease in control animals. Basal renal cortical blood perfusion was similar in both diabetic and control animals (318 ± 15, n = 8, vs. 339 ± 17 laser units, n = 7, respectively; NS), and it increased after injection of L-arginine and decreased after L-NAME injection in both groups. The decrease after L-NAME administration was largest in diabetic animals (Figs. 2 and 3). Before the injections, basal PO2 was lower in diabetic compared with control animals (33.6 ± 1.5 mmHg, n = 8, vs. 44.1 ± 3.2 mmHg, n = 7; P < 0.05) (Fig. 4). Injection of L-arginine did not significantly affect renal cortical PO2 in any of the two groups, whereas L-NAME decreased PO2 in control animals. There was a trend toward decreased PO2 after L-NAME administration also in diabetic animals, although this did not reach statistical significance. Mean arterial blood pressure was unaffected by l-arginine injection, but it increased as a result of L-NAME injection in both investigated groups (Table 1).

There was an approximately linear dependence of cortical PO2 on blood perfusion in both nondiabetic and diabetic animals (n = 7 and P < 0.05 in both groups, curve fit 0.621 in the nondiabetic group and 0.704 in the diabetic group) (Fig. 5). The relationship between renal cortical blood perfusion and PO2 was similar in both groups (multivariate ANOVA, P = 0.23).

The 4-week diabetic animals had a lower baseline GFR than control animals (Table 1). GFR in control animals (33.6 ± 1.5 mmHg, n = 8, vs. 44.1 ± 3.2 mmHg, n = 7; P < 0.05) (Fig. 4). Injection of L-arginine did not significantly affect renal cortical PO2 in any of the two groups, whereas L-NAME decreased PO2 in control animals. There was a trend toward decreased PO2 after L-NAME administration also in diabetic animals, although this did not reach statistical significance. Mean arterial blood pressure was unaffected by l-arginine injection, but it increased as a result of L-NAME injection in both investigated groups (Table 1).
L-arginine caused an increase, whereas L-NAME did not alter GFR. Baseline urinary flow rate was 10-fold higher in diabetic animals than in control animals (Table 1). In both groups, the urinary flow rate was unaffected by either of the two injections. Sodium excretion was unaffected by L-arginine injection in control animals, whereas injection of L-NAME resulted in increased sodium excretion (Table 1). None of the injections affected sodium excretion statistically in diabetic animals, although absolute values after L-NAME were more than twice the control level. Neither of the two injections had any effect on potassium excretion in either control or diabetic animals (Table 1). The plasma arginine concentration was significantly reduced in diabetic animals, whereas plasma concentrations of ADMA and SDMA were similar to control animals (Table 2).

**DISCUSSION**

The current study demonstrates substrate limitation for NO formation with concomitant lower NO concentration in the renal cortex of diabetic animals. These findings demonstrate a new mechanism for diabetes-induced alteration in renal oxygen metabolism and local blood flow regulation, which may have implications for the development of diabetes-induced vascular dysfunction.

So far, the reported effects of hyperglycemia on renal NO concentration and blood perfusion have been highly diverse (rev. in 21). There are several possible explana-
Renal NO concentration was measured in control and diabetic rats using microsensors. NO synthesis was found to be 48% lower in diabetic animals compared to nondiabetic animals, indicating a substrate limitation. The use of different animal models or human populations and different techniques for estimating the renal NO concentration may explain some of the discrepancies. Furthermore, the production and bioavailability of NO are not necessarily identical entities.

In the current study, Whalen-type microsensors were used to record the bioavailable NO concentration in vivo. Direct and online measurements of NO activity are achieved because of the design of the sensors. These highly sensitive sensors have shown their usefulness under various in vivo conditions. By using direct measurements, we observed that NO concentrations in the renal cortex appeared to be markedly changed in diabetic rats. Basal NO concentrations can be calculated with the assumption that L-NAME inhibits all, or close to all, NO synthesis, resulting in 87 nmol/l in nondiabetic animals and only 45 nmol/l in diabetic animals, or a 48% lower basal NO level. Injection of L-arginine resulted in an increased renal cortical NO concentration in both the nondiabetic and diabetic rats within minutes. Injection of L-arginine in vivo resulted in a substrate limitation or increased NOS activity, or a combination of both. NO synthesis is highly dependent on cellular transport of arginine and is therefore dependent on extracellular arginine availability. This availability is regulated by de novo arginine synthesis, cellular arginine transport, and the degradation rate by arginase.

Data are the means ± SE. Values are the means of the respective 20-min sampling period. *P < 0.05 when compared with the corresponding control period; †P < 0.05 when compared with L-arginine period in the same group; ‡P < 0.05 when compared with the corresponding period for control animals.

![Graph](image-url)

FIG. 5. Relationship between renal cortical blood perfusion and oxygen tension. Each group consist of seven animals with five to six measurements in each animal. ⊗ and dotted line, control animals; ◇ and solid line, diabetic animals.
It is well documented that the diabetic state induces increased cellular oxygen consumption (10,13,14,36). In the current study, basal renal cortical PO$_2$ i.e., that recorded before manipulation of NO production, was significantly lower in diabetic compared with control animals. This has also been reported during hypertension, another established state of increased radical production (37). We have previously reported (10) that decreased basal PO$_2$ in diabetic rats can be prevented by daily treatment with the radical scavenger $\alpha$-tocopherol throughout the course of diabetes, mainly because of prevention of the diabetes-induced increase in oxygen consumption by renal tubular cells (10). Renal PO$_2$ is influenced by two factors, namely the delivery of oxygen by the blood and the oxygen consumption within the renal tissue. Alterations in either of these factors are likely to change renal tissue PO$_2$ if no compensatory mechanism is activated. Because no difference in basal cortical blood perfusion was observed between nondiabetic and diabetic animals in the current study, it is most likely that the increase in renal metabolism, as previously reported (10,13), is the main mechanism responsible for the diabetes-induced decrease in renal cortical PO$_2$. Another possible explanation is a reduction in renal cortex capillary density in diabetic animals, but this seems unlikely because inhibition of NO decreased the renal cortical PO$_2$ in the nondiabetic animals to a similar level as that recorded in the diabetic animals, despite the more pronounced decrease in blood perfusion in the diabetic animals. Decreased bioavailability of NO will, however, not only cause vasoconstriction, but it will also result in increased oxygen consumption (5). Because the rate at which NO inhibits the mitochondrial respiration is an interplay between the concentrations of both NO and oxygen (7), the renal oxygen consumption is likely to increase more in the nondiabetic than in the diabetic animals after inhibition of NO because the NO decrease is proportionally larger in the control animals in relation to PO$_2$. The dependency of renal blood perfusion for PO$_2$ was similar in nondiabetic and diabetic animals (Fig. 5). We therefore conclude that the more pronounced decrease in PO$_2$ in the nondiabetic animals after L-NAME is caused by a higher baseline NO inhibition of the mitochondrial respiration rate. This accounts for the similar absolute PO$_2$ levels in the two investigated groups after NO inhibition.

Because of the duration of diabetes, the transient phase of glomerular hyperfiltration, which is present in this animal model during the first weeks after the induction of diabetes, had passed (38). Interestingly, Brown et al. (39) have shown that increased NO activity desensitizes the tubuloglomerular feedback mechanism, which could explain the increased GFR in the diabetic animals observed after L-arginine injection (40). The inverse mechanism could be responsible for the decreased GFR in the control animals observed after inhibition of NO production. There was a 10-fold higher urinary flow rate in the diabetic animals because of the osmotic diuresis. Altering NO production did not significantly affect the urinary flow rate in either of the two groups. The increased sodium excretion in the nondiabetic animals after NO inhibition is most likely an effect of pressure natriuresis. Even though the elevation in arterial pressure in the diabetic animals after L-NAME was similar to that of control animals, the increase in sodium excretion (twofold elevation) did not reach statistical significance due to large variation. It is, however, plausible that the mechanism underlying the higher sodium output after L-NAME in the diabetic animals

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**TABLE 2**

Plasma L-arginine, ADMA, and SDMA in control and 4-week diabetic animals

<table>
<thead>
<tr>
<th></th>
<th>L-arginine (µmol/l)</th>
<th>ADMA (µmol/l)</th>
<th>SDMA (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>127.9 ± 3.9</td>
<td>0.599 ± 0.017</td>
<td>0.328 ± 0.012</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td>79.7 ± 6.7*</td>
<td>0.581 ± 0.078</td>
<td>0.366 ± 0.056</td>
</tr>
</tbody>
</table>

Data are the means ± SE. *P < 0.05 when compared with control animals.
is similar to that in the control animals i.e., pressure natriuresis.

In conclusion, diabetic animals have a larger increase in the renal cortical NO concentration after injection of L-arginine compared with control animals which, together with the reduced plasma arginine concentration, demonstrate substrate limitation for NO synthesis by NOS. The decreased total renal NO concentrations in diabetic animals results in reduced tissue PO2 and altered regulation of blood perfusion. These findings demonstrate a new mechanism for diabetes-induced alteration in oxygen metabolism and blood flow regulation, which may have implications for the development of cardiovascular complications during diabetes, but it warrants further investigation.

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