Increased Renal Metabolism in Diabetes

Mechanism and Functional Implications

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The coupling between the Na^+/glucose cotransporter and Na^+-K^+-ATPase (NKA) described for epithelial cells (1) prompted us to study in rats with streptozocin-induced diabetes the effect of increased tubular glucose load on tubular Na^+ reabsorption, NKA-dependent O_2 consumption (QO_2), and NKA activity. Filtered glucose is mainly reabsorbed in the proximal tubuli via the phlorizin-sensitive Na^+/glucose cotransporter. In this study, the diabetic rats had a significantly higher renal blood flow (RBF), glomerular filtration rate (GFR), and Na^+ reabsorption than the control rats. Total renal QO_2 as well as QO_2 in cortical tissue, which consists mainly of proximal tubular cells, was significantly higher in diabetic than in control rats. The increase in tissue QO_2 was entirely caused by increased NKA-dependent QO_2. NKA activity, measured as rate of ATP hydrolysis, was increased in cortical tubular but not glomerular tissue from diabetic rats. Phlorizin treatment abolished the increase in NKA activity, Na^+ reabsorption, and QO_2, as well as the increase in RBF and GFR in diabetic rats. We conclude that diabetes is associated with increased renal O_2 metabolism secondary to the increase in coupled Na^+ reabsorption via the Na^+/glucose cotransporter and NKA. The increased oxygen consumption might contribute to the hyperperfusion and hyperfiltration in the diabetic kidney. *Diabetes* 43: 629–633, 1994

Diabetes is generally associated with increased glomerular filtration rate (GFR) and increased tubular sodium reabsorption (2–5). The enzyme Na^+-K^+-ATPase (NKA), which accounts for ~80% of the oxygen consumed by the kidney, yields energy to the transcellular transport of sodium in all tubular cells. Induction of experimental diabetes in rats results in a pronounced increase in renal tubular, albeit not glomerular, activity of NKA (6–11). This study examines the mechanism behind the increase in tubular NKA activity and sodium reabsorption in experimental diabetes and raises the question whether there might be a connection between the increase in tubular Na reabsorption and glomerular hyperfiltration.

Immediately after the onset of diabetes, the tubules are exposed to very high concentrations of glucose. Glucose is mainly reabsorbed via the Na^+/glucose cotransporters located in the brush-border membrane of proximal tubular cells (1). A high luminal glucose concentration might therefore enhance tubular sodium reabsorption (2), leading to increased intracellular Na^+ concentration and activation of NKA. To test this hypothesis, NKA activity was determined in rat proximal tubule cells exposed to various concentrations of glucose in the absence and presence of phlorizin, a specific inhibitor of the Na^+/glucose cotransporter. Because the results indicated that enhanced glucose uptake increases NKA-dependent oxygen consumption and thereby also total oxygen consumption, we next examined, in streptozotocin (STZ)-induced diabetic rats, whether increased activity of the Na^+/glucose cotransporter would result in increased renal oxygen uptake and might contribute to the increase in renal blood flow (RBF) and GFR.

RESEARCH DESIGN AND METHODS

The study was performed on male Sprague-Dawley rats (Alab, Sweden) fed a standard diet with 21% protein and provided water ad libitum. The rats were randomly allocated into control and experimental (diabetic) groups at 7 weeks of age.

Induction of diabetes. STZ (Zanosar, Upjohn, Kalamazoo, MI), 65–70 mg/kg, was injected into the tail vein. The animals received a daily subcutaneous injection of 2–4 U insulin (Ultratard Human, Novo Nordisk, Copenhagen, Denmark). Blood glucose concentration was measured regularly with a reflectance meter (Reflolux II, Boehringer Mannheim, Mannheim, Germany). STZ-administered rats with unfasting plasma glucose concentrations <16.5 mM at 48 h after injection were excluded from the study. Hyperglycemia in diabetic rats was apparent 12 h after STZ injection and remained stable throughout the whole observation period. At the time of studies, average blood glucose (mM) was significantly higher in diabetic compared with control rats (diabetic vs. control rats: 25.2 ± 0.5 vs. 6.8 ± 0.4 mM, $P < 0.001$). Experiments were conducted after 1–3 weeks of induction of diabetes, when the functional characteristics of the diabetic kidney, such as hyperfiltration, were already established.

Preparation of tubular segments. For preparation of tubular segments, the left kidney was perfused in situ via the aorta with a cooled modified Hanks' medium containing $CaCl_2$ (2 mM), bovine serum albumin (0.1%), and collagenase (0.05%). The outer cortex was dissected, cut into 3–4 mm long pieces, and incubated in the same solution at 35°C for 15 min and then for another 5 min while being continuously stirred. During incubation, the solutions were continuously exposed to 95% O_2 and 5% CO_2. The tubular segment suspension was subsequently cooled on ice and washed twice by Dulbecco's modified Eagle's medium (DMEM).

Preparation of tubular cells. Kidneys from two rats of the corresponding animal groups were pooled. The cortex was dissected from the medulla and thoroughly minced on ice with a razor blade. Thereafter it was incubated in DMEM containing collagenase (0.065%) at 37°C for 60 min. During incubation, the solutions were continuously exposed to 95% O_2 and 5% CO_2. After incubation, the cell suspension was cooled on ice and poured through graded filters with pore sizes of 80, 75, 53, and 38 mμ, respectively. The filtrate was kept on ice for 15 min to allow the tubular fragments to form a sediment. The remaining suspension, which

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Received for publication 14 June 1993 and accepted in revised form 5 January 1994.

GFR, glomerular filtration rate; NKA, Na^+-K^+-ATPase; RBF, renal blood flow; STZ, streptozotocin; DMEM, Dulbecco's modified Eagle's medium; A-V O_2, difference between arterial and venous oxygen content.

contained a population of 75–80% cortical cells, was washed three times in 20 ml DMEM without collagenase, and the cells were collected after a slow-spin (100 *g*) centrifugation for 4 min.

Isolation of glomeruli. Glomeruli were isolated by sieving through a series of stainless steel meshes with pore sizes of 150, 105, and 77 mμ, respectively, and by repeated washing with an excess volume of 0.9% NaCl. After centrifugation at 100 *g*, isolated glomeruli were resuspended in 2.0 ml buffer containing 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 and centrifuged again at 100 *g*. Thereafter the pellet was resuspended in 100 ml of the same solution and homogenized on ice. For each experiment, the renal cortex of one animal was used.

Measurement of NKA activity. To study the regulation of NKA activity in proximal tubule cells in diabetes, tubular NKA activity was measured by two different methods. In the homogenate studies, NKA was saturated with regard to Na^+, as well as with regard to the other major substrates, K^+ and ATP. The oxygen consumption studies were not carried out at V_{max} condition, because the tubular cells have an intracellular Na^+ concentration in the range of 10 to 20 mM, and V_{max} is reached at ~50 mM intracellular Na^+ concentration (12).

Measurement of NKA-dependent respiration. Tubular segments and tubular cells suspended in DMEM were transferred to a continuously stirred 3-ml chamber maintained at 37°C with a circulating water bath. Aliquots (500 μl) of the cell suspension were diluted with 2.5 ml incubation solution. The chamber was sealed with a Clark-type polarographic oxygen probe (Scientific Division, YSI, Yellow Springs, OH). To optimize mitochondrial respiration, butyric acid (10^{-3} M) was added just before the cells were put into the chamber. For the measurement of NKA-independent respiration ouabain (2×10^{-3} M) was added. All experiments were run in duplicate or triplicate. To assess the effect of different medium glucose concentrations on NKA-dependent respiration, the commercially available DMEM containing 5 mM glucose was supplemented with either 95 mM glucose or 95 mM mannitol. In some experiments, glucose was replaced by an equal concentration of methyl-α-D-glucopyranoside. This nonmetabolized analogue is actively transported in a sodium-dependent fashion at the same rate as D-glucose (13). In some protocols, amphotericin B (10 μg/ml) was added to the chamber.

Measurement of NKA activity in renal cortical homogenate. The renal cortex was homogenized on ice in 140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 buffer (pH 7.2) with a Teflon homogenizer (800 rpm, 5–6 strokes). Aliquots (10 μl) of cortical homogenates were permeabilized by 0.1% deoxycholate and incubated for 15 min at 37°C in a reaction mixture containing NaCl (140 mM), KCl (5 mM), $MgCl_2$ (5 mM), EGTA (1 mM), Tris-HCl (30 mM), Na_2ATP grade II (3 mM) (Sigma, St. Louis, MO), and [^{32}P]ATP (Du Pont-NEN, Boston, MA), 2–10 Ci/μmol in tracer amounts. The reaction was terminated by adding trichloroacetic acid (5%). For the determination of ouabain-insensitive (Mg-dependent) ATPase activity, NaCl and KCl were omitted, and 1 mM ouabain was added. The pH of both solutions was 7.4. The phosphate liberated by the hydrolysis of [^{32}P]ATP was separated by centrifugation at 8,000 *g* after absorption of the unhydrolyzed nucleotide on activated charcoal. The radioactivity was counted in a liquid scintillation counter. Incubation of the media alone gave the value for nonspecific ATP hydrolysis, which was subtracted from the values of total and ouabain-insensitive ATPase. In each experiment, NKA activity was calculated as the difference between the mean value for total and ouabain-insensitive NKA. The same procedure was applied for measurement of glomerular NKA activity.

Abundance of mRNA. The abundance of mRNA for NKA was determined as described previously (14). Briefly, 5 μg of total RNA was blotted into nylon filter and hybridized at 65°C in Amersham Rapid Hybridization buffer (Amersham, U.K.). Samples were blotted in triplicate, and the corresponding dots were scanned by laser densitometer three times. The mean was calculated and then normalized to a standard (control kidney homogenate) to account for variations between different filters. Quantitative loading of mRNA samples was evaluated by monitoring the expression of actin mRNA for each blot. The β_1 cDNA is the *Hin*dIII-*Pst* I fragment (nucleotides 913–1,184), and α_1 is a full-length cDNA. Actin cDNA was purchased from Clontech (Palo Alto, CA).

Renal function. GFR was measured as the clearance of inulin (Laevosan Gesellschaft, Linz, Austria). Inulin was diluted in normal saline (5%) and infused at a rate of 1 ml · 100 g body $wt^{-1} \cdot h^{-1}$. The infusion was preceded by a priming dose of the infusate, 1 ml/100 g body wt. After a 60-min equilibration, urine was collected from tubes placed in both ureters and sampled for two (45–60 min) periods (15). Chemical analysis of inulin in blood and urine was performed by a standard laboratory method (16). In experiments in which the effect of phlorizin was tested, the drug was administered after the first clearance period.

TABLE 1
NKA activity measured as ouabain-sensitive O_2 consumption in proximal tubular segments of diabetic and control rats

		O_2 consumption ($\mu l\ O_2 \cdot mg\ protein^{-1} \cdot h^{-1}$)	
	n	Total	Ouabain-inhibitable
Diabetic rats	12	120.0 ± 10.2*	42.0 ± 3.7†
Control rats	13	91.0 ± 6.9	26.4 ± 2.6

Data are means ± SE. * $P < 0.05$ compared with control rats. † $P < 0.01$ compared with control rats.

RBF was measured with the microsphere technique as described previously (17), using carbonized microspheres, 15 ± 2 μm in diameter, labeled with ^{51}Cr. The microspheres were injected, and the reference blood samples were withdrawn at the same speed, 0.3 ml/min. This withdrawal rate allows the trapping of enough spheres in the reference sample without producing any observable effects on the cardiovascular system. After withdrawal of the reference sample, the kidneys were removed and weighed. The radioactivity in tissues and in the reference blood samples was analyzed in a γ-spectrometer (Packard, Downers Grove, IL).

The difference between arterial and venous oxygen content (A-V O_2) was determined by the simultaneous measurement of oxygen tension in the right carotid artery and the left renal vein by an oxygen electrode (ABL3 radiometer, Acid-Base Laboratory, Copenhagen, Denmark).

Other analytical methods. Sodium in serum and urine was measured by flame photometry. Protein content of isolated glomeruli, tubular cells, and cortical homogenate was determined by the Bio-Rad (Richmond, CA) method (18).

Drugs and chemicals were purchased from Sigma unless otherwise stated and were of the highest chemical purity. Phlorizin was administered either chronically in a dose of 400 $mg \cdot kg^{-1} \cdot day^{-1}$ divided into three equal doses and injected subcutaneously for 5 days or infused intravenously in a dose of 400 $\mu g \cdot kg^{-1} \cdot min^{-1}$ after an appropriate priming.

Statistical analysis. All data are given as means ± SE. Statistical analysis was performed with paired and unpaired Student's *t* test and analysis of variance when appropriate.

RESULTS

In vitro studies. NKA activity measured as the rate of ATP hydrolysis in renal cortical homogenate was higher in the diabetic than in the control rats (diabetic vs. control rats: 24.4 ± 1.1 vs. 15.5 ± 0.9 $\mu M\ P_i \cdot mg\ protein^{-1} \cdot h^{-1}$, $n = 6$ and 7, respectively, $P < 0.001$). In contrast, a significant decrease in glomerular NKA activity (9.7 ± 1.3 vs. 15.0 ± 1.2 $\mu M\ P_i \cdot mg\ protein^{-1} \cdot h^{-1}$, $P < 0.02$ was observed in diabetic vs. control rats). NKA activity was also measured as ouabain-inhibitable oxygen consumption in renal cortical cells and was higher in the diabetic than in the control rats. Total oxygen consumption was also increased in the renal cortical cells from the diabetic rats. This increase was entirely attributable to the increase in ouabain-sensitive oxygen consumption (Table 1).

The expression of renal cortical NKA mRNA, measured 2, 3, 4, 7, and 21 days after the induction of diabetes, was similar in diabetic and control rats for both subunits. The mRNA α_1 was 0.93 ± 0.1 relative unit in control and 0.90 ± 0.05 relative unit in diabetic rats. The mRNA β_1 was 0.81 ± 0.06 relative unit in control and 0.77 ± 0.06 relative unit in diabetic rats.

To evaluate the relationship between stimulation of the Na^+/glucose cotransporter and the activation of NKA, ouabain-sensitive oxygen consumption was determined in cortical tubule cells from control rats in the presence of two

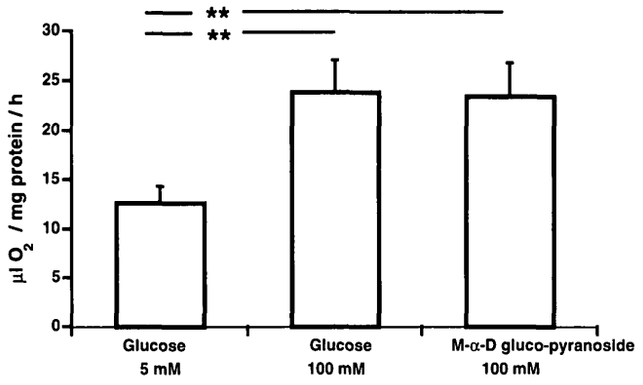

FIG. 1. NKA activity measured as ouabain-sensitive O_2 consumption (µl O_2/mg protein/h) in proximal tubular cell suspension from control rats incubated in media with 5 mM (n = 7) and 100 mM (n = 6) glucose concentrations and with the nonmetabolized glucose analogue (methyl-α-D-glucopyranoside, n = 6). For each experiment, kidneys from two rats were pooled. **$P < 0.01$.

different glucose concentrations, 5 and 100 mM, respectively. The higher glucose concentration significantly increased O_2 consumption in the control cells (100 vs. 5 mM glucose: 23.8 ± 3.3 vs. 12.6 ± 1.6 µl $O_2 \cdot$ mg protein$^{-1} \cdot h^{-1}$). A similar increase was obtained when the cells were exposed to the nonmetabolized glucose analogue, methyl-α-D-glucopyranoside, which uses the same Na^+-dependent transporter as glucose at the apical membrane of the proximal tubular cell (Fig. 1). The ionophore amphotericin B significantly increased oxygen consumption in tubular cells from control animals incubated in both 5 mM (39.1 ± 1.6 vs. 51.6 ± 3.0 µl $O_2 \cdot$ mg protein$^{-1} \cdot h^{-1}$) and 100 mM (42.8 ± 4.4 vs. 61.0 ± 7.8 µl $O_2 \cdot$ mg protein$^{-1} \cdot h^{-1}$) media. In the presence of amphotericin, oxygen consumption in cells incubated in 100 mM glucose was still significantly higher ($P < 0.02$) than that in cells incubated in the 5 mM glucose medium.

In vivo studies. Within a few days after STZ administration, the rats exhibited the typical early manifestations of diabetes, and this effect on renal function was sustained for at least 3 weeks. GFR in diabetic rats was increased by 29% (diabetic vs. control rats: 2.93 ± 0.11 vs. 2.28 ± 0.13 ml/min, $P < 0.01$). RBF was increased out of proportion (54%) to the increase in GFR (diabetic vs. control rats: 11.7 ± 0.6 vs. 7.6 ± 0.7 ml/min, $P < 0.001$). Tubular sodium reabsorption was increased by 37% (diabetic vs. control rats: 411 ± 15 vs. 310 ± 22 µmol/min, $P < 0.01$). Because simultaneous determinations of A-V O_2 difference and RBF could not be performed for technical reasons, we calculated renal oxygen consumption by multiplying each value for A-V O_2 difference with the mean value for RBF in each group. The estimated values for oxygen consumption were 58.5 ± 2.9 and 29.8 ± 2.5 ml/min ($P < 0.001$) in the diabetic and control groups, respectively. Variations in RBF and oxygen consumption were negligible (1–3%) when measurements performed at different time points (from 4 days to 3 weeks) were compared.

We next determined the effect of inhibition of the Na^+/glucose cotransporter on renal function. For this purpose, the rats were infused with phlorizin in a dose of 400 µg $\cdot$ kg$^{-1} \cdot$ min^{-1} (see METHODS). In the clearance studies, a control period was obtained for comparison before the infusion was started. Treatment with phlorizin resulted in a small, but nonsignificant, increase in diuresis (0.0329 ± 0.0041 vs. 0.0475 ± 0.0041 ml/min, NS) and in an increase of urinary glucose excretion (0.14 ± 0.02 vs. 0.31 ± 0.02 mmol/min, $P < 0.001$) in the diabetic rats. The latter resulted in a significant decrease of blood glucose level, although hyperglycemia was well maintained (22.3 ± 1.1 vs. 14.0 ± 0.85 mM, $P < 0.001$). The hematocrit did not change (47.2 ± 0.82 vs. 49.1 ± 0.60, NS). Although this does not completely rule out the possibility that osmotic diuresis during phlorizin treatment might, via extracellular fluid contraction, contribute to the decrease in RBF and GFR, other explanations (see DISCUSSION) seem to be more likely. In control rats, initial glucose excretion was negligible but rose significantly after phlorizin infusion (0.0005 ± 0.0002 vs. 0.15 ± 0.03 mmol/min, $P < 0.001$). Infusion of phlorizin into diabetic animals promptly normalized the increased tubular sodium reabsorption and renal oxygen consumption (Fig. 2*A* and *B*). Phlorizin did not alter the rate of Na^+ reabsorption and had no effect on renal oxygen consumption in the control rats. Phlorizin infusion also significantly decreased RBF and GFR in the diabetic rats (Fig. 2*C* and *D*).

Five days of phlorizin administration significantly decreased elevated NKA activity measured in cortical kidney homogenate as ATP hydrolysis. As shown in Fig. 3, NKA activity in phlorizin-treated diabetic animals was indistinguishable from that of the control rats.

DISCUSSION

NKA plays a pivotal role for tubular sodium transport. This enzyme will, via active countertransport of Na^+ and K^+ across the basolateral membrane, create the electrochemical gradient that allows Na^+ to enter the cell via a variety of co- and countertransporters such as the Na^+/glucose cotransporter. The Na^+/glucose cotransporter is, like most of the Na^+/amino acid transporters, located apically in the proximal tubule cell (1). Under basal conditions, NKA is not saturated with regard to Na^+. An increased flux of Na^+ into the proximal tubular cells should lead to increased intracellular sodium content and activation of the sodium pump (19–21). The same mechanism might be responsible for the increase in NKA activity after the cells are loaded with the ionophore amphotericin B. The ouabain-sensitive oxygen consumption, an index of NKA activity, was increased in tubular cells from diabetic rats. Exposure of cells from control rats to a high glucose concentration also increased the ouabain-sensitive oxygen consumption. This increase could be attributed to increased influx of Na^+ via the Na^+/glucose cotransporter because a high concentration of the nonmetabolized glucose analogue had an effect on the ouabain-sensitive oxygen consumption as high glucose concentration. Thus, our observations confirm the concept that passive influx of Na^+ via the Na^+/glucose cotransporter is coupled to active extrusion of Na^+ by the Na^+ pump, and suggest that the increase in renal tubular NKA activity that we and others (5–9) have observed is a result of increased activity of the Na^+/glucose cotransporter. In support of this hypothesis, we demonstrated that in diabetic rats phlorizin treatment decreased NKA activity.

It has been postulated that a longstanding increase in intracellular Na^+ content might lead to increased abundance and/or increased availability of the functioning units of NKA. The finding that NKA activity was increased in diabetic rats that had not been treated with phlorizin supports this hypothesis. The lack of increase in mRNA implies that the increase in activity is caused by a translational/posttranslational rather than a transcriptional effect (22–24).

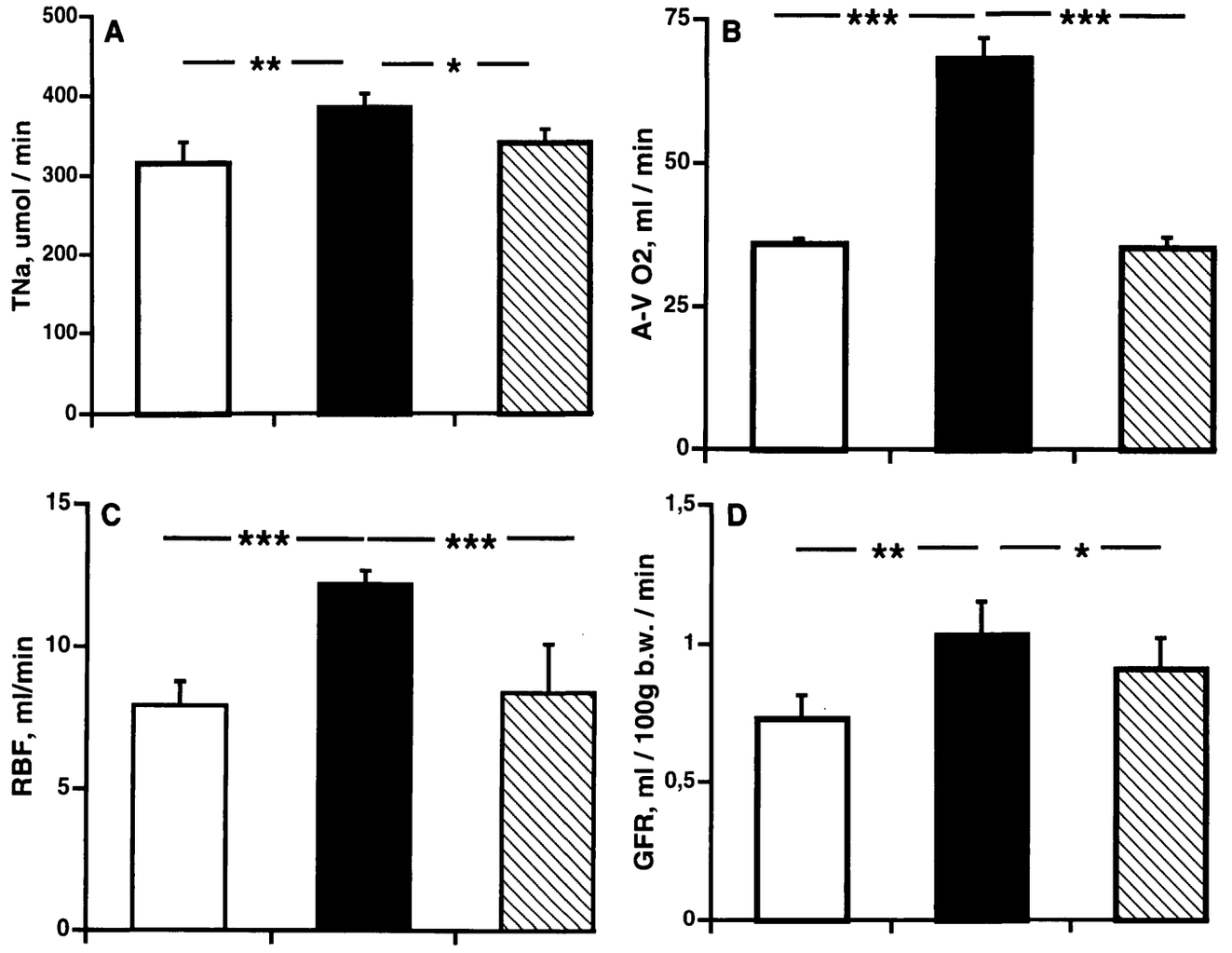

FIG. 2. Tubular sodium reabsorption (T_{Na}) (*A*), A-V O_2 (*B*), RBF (*C*), and GFR (*D*) in control rats (□), STZ-induced diabetic rats (■), and phlorizin-treated STZ-induced diabetic rats (▨). Phlorizin was infused during the experiments in a dose of 400 $\mu g \cdot kg^{-1} \cdot min^{-1}$ after priming. Phlorizin did not have any measurable effect on renal functional parameters in control rats. Studies were performed in 4–7 rats in each group. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

Hyperperfusion and hyperfiltration are typical findings in patients with poorly controlled diabetes and appear to be causally related to hyperglycemia. Hyperfiltration is more often present in patients with poorly controlled diabetes, than in those with a good glycemic control (25,26), and improvement of the glycemic control by insulin treatment reduces or even normalizes hyperfiltration both in patients and in experimental animals (27,28). The mechanism by which hyperglycemia causes hyperperfusion remains unclear, despite numerous studies of this subject. It has been suggested that hyperglycemia might result in the release of hormones that have a vasodilatory effect on the renal vascular bed. However, experimental studies have not given unequivocal evidence that any of the hormones released by hyperglycemia play a critical role for the induction of diabetic hyperfiltration (29). Here we show that inhibition of the coupled Na^+/glucose transporter by phlorizin promptly attenuated or abolished the hyperfiltration and hyperperfusion in the diabetic kidney. A decrease in the blood glucose level could not be responsible for the cessation of hyperfiltration, because a similar level of hyperglycemia under normal conditions, i.e., without phlorizin treatment, is sufficient to increase GFR in diabetic animals (30). Inhibition of the Na^+/glucose cotransporter also decreased tubular Na^+ reabsorption and renal oxygen consumption. Because the activity of the Na^+/glucose cotransporter is coupled to the activity of NKA, we attribute the decrease in renal oxygen consumption to a decrease in NKA activity. NKA-mediated transcellular

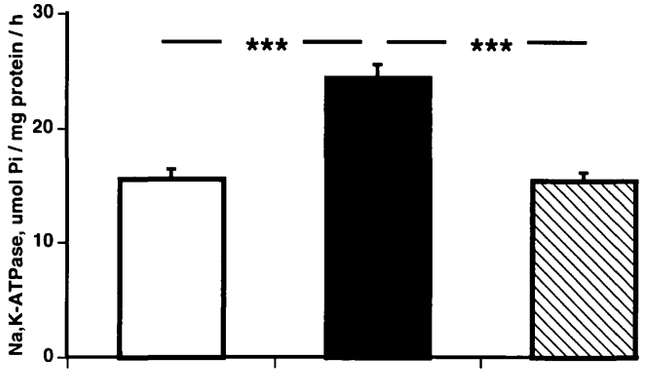

FIG. 3. NKA activity, measured as the rate of ATP hydrolysis in renal cortical homogenate of diabetic rats. Phlorizin was administered chronically in a dose of 400 $mg \cdot kg^{-1} \cdot day^{-1}$ divided into three equal doses and injected subcutaneously for 5 days before the experiments: control rats (□, $n = 7$), untreated diabetic rats (■, $n = 6$), and phlorizin-treated diabetic rats (▨, $n = 4$). $^{***}P < 0.001$.

Na^+ transport accounts for ~80% of the energy consumed by the kidney. In diabetic rats, oxygen consumption of proximal tubule cells was increased approximately twofold. We speculate that the diabetic kidney will require a higher blood supply, i.e., elevated RBF, to meet this increased oxygen demand. The same phenomenon might explain the increase in GFR that occurs after amino acid infusion or a high protein meal (31), procedures that will lead to increased filtration of amino acids. Amino acids are taken up in the proximal tubule cell in a Na^+-coupled way, and high protein intake has also been shown to increase the ouabain-sensitive oxygen consumption in this cell type (32). The hormonal mediators of renal vasodilation on amino acid infusion or hyperglycemia-induced increased Na^+ reabsorption because of the diabetic state, are not fully clarified yet. In a recently published work, Williamson et al. (33) suggested that hyperglycemia, referred to as pseudohypoxia because tissue partial oxygen pressure is normal, mimics the effect of hypoxia on the vascular bed. An increased ratio of free NADH/NAD^+, appearing under both hypoxic and hyperglycemic conditions, results in an increased lactate/pyruvate ratio. It has been shown previously that lactate increases RBF and GFR (34). However, the effect of other hormones and vasoactive substances (29), among others endothelium-derived factors such as nitric oxide (35), should also be taken into consideration.

In conclusion, the elevation in Na^+/glucose cotransport activity and concomitant increase in NKA activity might, by a still unidentified mechanism, be an important contributor to the increased RBF and GFR in diabetes. The finding that inhibition of glucose uptake via the Na^+/glucose cotransporter by phlorizin resulted in a significant decrease in tubular sodium reabsorption and renal oxygen uptake in the diabetic animals is consistent with this hypothesis.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swedish Medical Research Council (Project 03644).

We thank M. Agrén and E. Zettergren for expert technical assistance.

Parts of this study were presented at the 25th annual meeting of the American Society of Nephrology, Baltimore, Maryland, 15–18 November 1992.

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