Delayed recovery of renal regional blood flow in diabetic mice subjected to acute ischemic kidney injury

Haikun Shi, Daniel Patschan, Tracy Epstein, Michael S. Goligorsky, and Joseph Winaver

Departments of Medicine, and Renal Research Institute, New York Medical College, Valhalla, New York; and Department of Physiology and Biophysics, Technion: Israel Institute of Technology, Haifa, Israel

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Shi H, Patschan D, Epstein T, Goligorsky MS, Winaver J. Delayed recovery of renal regional blood flow in diabetic mice subjected to acute ischemic kidney injury. Am J Physiol Renal Physiol 293: F1512–F1517, 2007.—Ischemic acute kidney injury in experimental diabetes mellitus (DM) is associated with a more severe deterioration in renal function than shown in nondiabetic animals. We evaluated whether the early recovery phase from acute kidney injury is associated with a more prolonged and sustained decrease in renal perfusion in diabetic mice, which could contribute to the impaired recovery of renal function. Perfusion to the renal cortex and medulla was evaluated by laser-Doppler flowmetry in 10- to 12-wk-old anesthetized mice with type 2 DM (db/db), heterozygous mice (db/m), and nondiabetic (control) mice (C57BL/6J). After baseline measurements were obtained, the right renal artery was clamped for 20 min followed by reperfusion for 60 min. The data demonstrated that, in all three groups studied, the reperfusion phase was characterized by a significant increase in the medullary-to-cortical blood flow ratio. Moreover, during recovery from ischemia, there was a marked prolongation in the time (in min) required to reach peak reperfusion in the cortex (db/db: 20.7 ± 4.0, db/m: 12.92 ± 1.9, C57BL/6J: 9.3 ± 1.3) and the medulla (db/db: 20.8 ± 3.2, db/m: 12.88 ± 1.89, C57BL/6J: 11.2 ± 1.2). Additionally, the slope of the recovery phase was lower in db/db mice (cortex: 61.9 ± 23.1%/min, medulla: 16.3 ± 3.6%/min) than in C57BL/6J mice (cortex: 202.2 ± 41.6%/min, medulla: 42.1 ± 7.2%/min). Our findings indicate that renal ischemia is associated with a redistribution of blood flow from cortex to medulla, not related to DM. Furthermore, renal ischemia in db/db mice results in a marked impairment in reperfusion of the renal cortex and medulla during the early postischemic period.

Diabetes mellitus; acute kidney injury; ischemia-reperfusion; laser-Doppler flowmetry

RENAL ISCHEMIA-REPERFUSION injury is characterized by an endothelial dysfunction that plays a key role in the early initiation and in the extension phase of ischemic renal failure (2, 9, 10, 23). The endothelial cell dysfunction is thought to be associated with increased generation of reactive oxygen intermediates and overexpression of a variety of inflammatory mediators, which may cause impairment in vascular competence and further impede blood supply to the ischemic kidney (2, 3, 5, 10, 23, 24). Indeed, alteration in renal microcirculation due to endothelial cell swelling following renal ischemia, known as the phenomenon of “no-reflow,” was already described in the early 1970s (8, 29). However, because of its transient nature, the no-reflow phenomenon was not considered to play a cardinal role in the pathogenesis of ischemic renal damage. The increasing recognition of the important role of endothelial dysfunction in the pathogenesis of acute renal failure (ARF) has led to reexamination of this concept. Indeed, a more recent study that used intravital videomicroscopy techniques has demonstrated profound alterations in peritubular capillary perfusion pattern in the early postocclusive phase in rats subjected to renal artery clamping (35). These alterations included a temporary loss of patency in portions of peritubular microcirculation combined with a shift in capillary blood flow from orthograde to retrograde direction. Also, measurement of erythrocyte velocity after release of renal artery occlusion exhibited a partial recovery followed by a significant and sustained deceleration (35).

Diabetes mellitus is another clinical situation characterized by features of endothelial dysfunction and oxidative stress (1, 4, 11). Thus it is reasonable to assume that renal ischemia in diabetes may result in a more severe form of renal injury. Indeed, increased susceptibility of the kidney to ischemic injury has been previously reported in several experimental models of diabetes mellitus, as well as in several studies in patients with diabetes (12, 21, 22, 27, 31, 34). However, the mechanisms underlying the enhanced vulnerability of the kidney to ischemia in diabetes have not been clearly established. We predicted that the “diabetic milieu” could exacerbate renal damage induced by ischemia-reperfusion in part by augmentation of endothelial dysfunction, which could cause a further deterioration in capillary blood flow during the early reperfusion phase. In the present study, we evaluated by laser-Doppler flowmetry the early postocclusive changes in renal regional perfusion in db/db mice, an experimental model of type 2 diabetes mellitus, compared with heterozygous db/m and non-diabetic C57BL/6J control mice. Our findings demonstrate a marked delay in renal perfusion, to both the renal cortex and outer medulla, during the early postischemic phase in diabetic mice.

METHODS

Studies were performed in 10- to 12-wk-old male C57BL/6J mice, db/db diabetic mice and db/m heterozygous mice (Jackson Laboratories, Bar Harbor, ME). Animals were maintained on regular rodent chow and water ad libitum before experiments. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, no. 85-23, revised 1996). All protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College. Two

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days before the experiment, control (n = 9), heterozygous (n = 9), and diabetic (n = 10) mice were transferred to individual metabolic cages for measurement of 24-h urine flow rate and albumin excretion rate.

Mice were anesthetized by intraperitoneal injection of 2% solution of α-chloralose (3.75 μg/g body wt) and 10% urethane (11.25 μg/g body wt). The right kidney was exposed through a flank incision, and the renal artery and vein were carefully separated from the perirenal fat under binocular magnification. A loose silk suture (2-0) was placed around the renal vessels. After surgical preparation, the kidney was placed in a Plexiglas cup and immobilized without exerting tension on the renal vessels. The surface of the kidney was gently flushed by 0.9% saline solution. Cortical and medullary blood flows (CBF and MBF, respectively) were measured simultaneously by a dual-channel laser-Doppler flowmeter (Periflux 5000, Perimed) using two calibrated needle probes with a fiber separation of 0.25 mm (model 403, Perimed). For measurement of CBF, the probe was placed perpendicularly to the surface of the cortex, and MBF was measured by a probe inserted into the outer medulla at a depth of 3–4 mm. The position of the probe in the outer medulla was verified at the end of each experiment by dissection of the kidney. After a short equilibration period, steady-state baseline recordings of CBF and MBF were obtained in control and diabetic mice for ~15 min (baseline period). The suture around the renal vessels was then tightened by a clamp to occlude blood flow for 20 min (ischemic phase). The clamp was subsequently released to restore blood perfusion to the ischemic kidney, and recordings of CBF and MBF were obtained for an additional 40–60 min (reperfusion period). The reperfusion period was characterized by an early peak in which tissue perfusion reached a maximal value, followed by a more prolonged and sustained steady-state phase (Fig. 1).

Electrical signals of both probes were digitized and recorded in real time and analyzed by Perisoft for Windows software (Perimed). CBF and MBF were calculated by measuring the velocity of the moving red blood cells multiplied by their concentration and expressed in perfusion units.

The following parameters were analyzed (see Fig. 1): 1) MBF-to-CBF ratio during baseline, peak, and steady-state reperfusion phases; 2) percent change of peak and steady-state reperfusion values from baseline values of CBF and MBF, calculated as average peak or steady-state value divided by average baseline value and expressed as percent change from baseline; 3) time to peak, i.e., time (in min) elapsing from release of clamp to peak reperfusion value of CBF and MBF; 4) percent change during recovery calculated by the formula (peak reperfusion value/minimal value during ischemic phase) × 100; 5) slope of recovery phase, calculated by dividing the percent change during recovery value by the time to peak and expressed as percent change per minute.

To verify that the diabetic mice actually developed a more severe kidney injury, additional studies were performed in db/db and db/m mice. These mice were anesthetized by intraperitoneal injection of ketamine plus xylazine, and their kidneys were exposed through a midabdominal incision. Both the right and left renal arteries were clamped for 20 min with microserrefines (Fine Science Tools, Foster City, CA). After release of the clamps, the abdominal wall was sutured and the mice were returned to their metabolic cages for a follow up of 48 h. Most of the animals were treated after the operation by a single injection of cefazolin (50 mg/kg im) and butorphanol tartrate (0.2 mg/kg im) to prevent infection and to relieve postoperative pain. Mice that survived for 48 h were anesthetized, and blood samples were taken by direct puncture of the heart for analysis of serum creatinine concentration.

Finally, because arterial pressure was not measured during the experiments, it was necessary to exclude any influence of changes in blood pressure on the obtained results. Thus additional groups of db/m (n = 6) and db/db mice (n = 3) were anesthetized and prepared identically to the original protocol of the laser-Doppler measurements. Systolic, diastolic, and mean arterial pressure (MAP) measurements were obtained by the tail-cuff technique with the noninvasive blood pressure monitor-8 (Columbus Instruments) in trained mice, averaging five measurements per time point. Measurements were obtained during the baseline period before renal arterial clamping, during renal ischemia, and after release of the clamp.

Chemical analysis. Glucose concentrations were measured in blood samples taken from the tail vein of conscious animals by a commercial glucometer (Ascencia Contour, Bayer HealthCare). Serum creatinine was measured by a colorimetric method with a commercially available kit (Raichem, San Diego, CA) according to the manufacturer’s protocol. Urine albumin concentration was evaluated with the Albuwell M kit, and urine creatinine was analyzed with the Creatinine well M kit, and urine creatinine was analyzed with the Creatinine Companion (both from Exocell, Philadelphia, PA).

Statistics. Statistical evaluation of the data was performed by ANOVA for repeated or unreplicated measures, as appropriate. For post-ANOVA evaluation, Dunnett’s test was used for comparison of baseline with peak and steady-state reperfusion values in each group and Tukey’s multiple comparisons test for group comparisons. *P < 0.05 was considered statistically significant. Data are presented as means ± SE.

RESULTS

At the age of 10–12 wk, diabetic mice displayed marked obesity [43.1 ± 0.7 g body wt, compared with the heterozygous (27.6 ± 0.6 g body wt) and control (28.6 ± 0.99 g body wt) mice]. Blood glucose levels in diabetic mice were significantly higher than levels in heterozygous mice (569.4 ± 14.9 vs. 158.3 ± 26.6 mg/dl) and control mice (99.5 ± 5.7 mg/dl). Likewise, urinary albumin-to-creatinine ratio was significantly higher in db/db mice (1.87 ± 0.48) than in db/m (0.33 ± 0.15) or C57BL/6J mice (0.02 ± 0.002).

Induction of bilateral renal ischemia was associated with a high mortality rate in the db/db mice but not in the heterozygous db/m mice. Thus 15 of 24 db/db mice (62.5%) died within 48 h after bilateral renal artery clamping. However, all of the db/m heterozygous mice (21/21) survived after an identical surgical procedure. In line with this finding, serum creatinine was significantly higher 48 h after surgery in the db/db mice that survived than in the db/m mice (1.44 ± 0.23 vs. 0.65 ± 0.18 mg/dl, respectively; *P < 0.02).

MBF/CBF results, before and after occlusion of renal artery, are shown in Fig. 2. During the baseline period, MBF/CBF was

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**Fig. 1.** Representative tracing of renal cortical perfusion throughout an experiment. The baseline, renal ischemia, early peak, and steady-state phases of reperfusion are shown, together with the various parameters examined.
of similar magnitude in control, heterozygous, and diabetic mice (0.35 ± 0.03, 0.42 ± 0.03, and 0.37 ± 0.04, respectively). Moreover, after release of renal arterial clamp, during the early peak reperfusion phase, MBF/CFB increased significantly in control mice (0.57 ± 0.08), db/m mice (0.52 ± 0.05), and db/db mice (0.73 ± 0.1) and remained significantly elevated over baseline values also during the steady-state phase (control: 0.57 ± 0.07; db/m: 0.51 ± 0.05; db/db: 0.71 ± 0.11; Fig. 2). This indicates that the recovery from renal ischemia is associated with a redistribution of intrarenal blood flow from cortex to medulla in all three experimental groups of mice. As shown in Fig. 3, the redistribution of intrarenal blood flow during the early peak recovery phase was due to a decrease in CBF (control: −16.9 ± 11.3%, db/m: −11.0 ± 3.7%, and db/db: −29.2 ± 10.3%) associated with an increase in MBF (control: 23.9 ± 6.4%, db/m: 8.0 ± 7.0%, and db/db: 31.1 ± 16.6%). Thus the redistribution of intrarenal blood flow, in control, heterozygous, and diabetic mice, was a consequence of renal ischemia per se, unrelated to the presence or absence of the diabetic milieu.

Figure 4 depicts the time interval (in min) required to reach maximal reperfusion (peak value) in the renal cortex and medulla after the renal clamp was released in control, heterozygous, and diabetic mice. As shown, this period was significantly prolonged in the db/db mice vs. that shown in control mice, in both the renal cortex (20.7 ± 4.0 vs. 9.3 ± 1.3 min, respectively; P < 0.02) and renal medulla (20.8 ± 3.2 vs. 11.2 ± 1.2 min, respectively; P < 0.01). The time to peak reperfusion results in the renal cortex (12.92 ± 1.91 min) and medulla (12.88 ± 1.89 min) of the heterozygous group were not statistically different from the results in control mice.

The changes in renal perfusion after release of the renal clamp and restoration of blood flow to the cortex and medulla are shown in Fig. 5. In control mice, there was an increase of 1,512 ± 219% in cortical perfusion, estimated by the intensity of the laser-Doppler signal during peak reperfusion divided by this parameter during renal arterial clamping. This value was significantly higher than the increase of 605 ± 92% observed in db/db mice (P < 0.01). The percent change in medullary perfusion, although numerically higher in control mice than in diabetic animals, did not reach statistical significance (control mice: 437.4 ± 62.7%, diabetic mice: 263.7 ± 52.4%). The percent change during the recovery phase of the heterozygous mice reached intermediate values in both the cortex (1,055 ± 172%) and the medulla (333.1 ± 37.9%) (Fig. 5). In line with these findings, the calculated slope of recovery was significantly lower in diabetic than in control mice (Fig. 6). This decrease was observed in both the cortex (control: 200.2 ± 41.6%/min, db/db: 61.9 ± 23.1%/min; P < 0.05) and the renal medulla (control: 42.1 ± 7.2%/min, db/db: 16.3 ± 3.6%/min; P < 0.05). In the db/m group, the slope of recovery was of
intermediate value in both the renal cortex (107.6 ± 28.2%/min) and the medulla (31.4 ± 5.0%/min).

Finally, MAP measured by the tail-cuff technique tended to be higher in the db/db group and gradually decreased in both db/db and db/m mice during the follow-up period (Table 1). However, MAP values after release of the renal artery clamp were very similar in both groups, and there was no evidence of hemodynamic instability during the follow up in any of the groups.

DISCUSSION

The findings of the present study provide novel information on the early postischemic phase in diabetic mice subjected to acute renal ischemic injury. Our data demonstrate that the reperfusion after renal ischemia is markedly delayed in mice with type 2 diabetes compared with that shown in nondiabetic control mice. This delayed recovery of renal blood flow was evidenced by all three parameters analyzed: time to peak reperfusion, the percent change of signal intensity from renal occlusion to peak of reperfusion, and the slope of reperfusion curve (Fig. 1). Moreover, the delayed recovery during the reperfusion phase occurred in both the renal cortex and the medulla of diabetic mice. Finally, renal ischemia-reperfusion injury was associated with a redistribution of intrarenal blood flow from the renal cortex to the medulla. It is noteworthy that similar changes in MBF/CBF occurred in the diabetic, heterozygous, and nondiabetic animals, suggesting that the redistribution of intrarenal blood flow is a consequence of the ischemia per se, unrelated to the presence or absence of diabetes.

Also of interest is the finding that, in several parameters examined, the db/m heterozygous mice occupied an intermediate position between the control and db/db mice. Under basal conditions, db/m mice are not obese and their blood glucose...
Admittedly, neither a cause-and-effect relationship nor a mechanism for the delay in tissue reperfusion could be explained by the differences in several parameters between the db/m and db/db mice. In our study, although the differences in several parameters between the db/m and db/db did not reach statistical significance, when the db/db mice and the “wild-type” C57BL/6J mice were compared, the difference became highly significant. It is therefore possible that some of the renal vascular disturbances are already present to a slight extent in the heterozygous state. Likewise, it is also possible that heterozygous mice are more prone to the development of hyperglycemia and other metabolic disturbances when exposed to stressful conditions like anesthesia. Indeed, their baseline blood glucose level was higher than that of C57BL/6J mice.

In a previous study from this laboratory (35), in which cortical perfusion was analyzed by intravital microscopy at a resolution of a single capillary, the reperfusion phase after renal ischemia was associated with major disturbances in peritubular capillary blood flow. These included a sustained deceleration of erythrocyte velocity and a temporary loss of patency in parts of peritubular microcirculation with a consequent shift in capillary blood flow from orthograde to retrograde direction. In the present study, the more integrative laser-Doppler technique was used to analyze overall alterations in regional perfusion of capillary beds. The findings of the present study provide further evidence for a significant delay in capillary reperfusion in both the renal cortex and the renal medulla of diabetic mice subjected to renal ischemia. This suggests that the impediment in peritubular microcirculation induced by ischemia may be augmented by the diabetic milieu, leading to a more prolonged recovery from renal ischemia. Such a prolongation of reperfusion period, i.e., doubling the time required to reach peak reperfusion in both the renal cortex and the medulla, could have significant pathophysiological consequences. In particular, this delay in blood supply to the kidney could result in a diminished tissue reoxygenation and at the same time could enhance oxidative and nitrosative stress (3, 24). This combination could serve as a trigger for the more pronounced cellular damage observed after renal ischemia in diabetic animals. Indeed, db/db mice subjected to 20 min of bilateral renal ischemia displayed very high mortality rates and a significantly higher serum creatinine level already 48 h after the ischemia than the db/m mice. This might suggest that the presence of diabetic milieu could aggravate the consequences of renal ischemia, leading to a more severe deterioration in renal function. However, although our study demonstrates a delayed recovery phase and an increased susceptibility for kidney failure in diabetic mice, these events could be unrelated. Admittedly, neither a cause-and-effect relationship nor a mechanismic link is provided by our data, and further studies are required to elucidate the exact interrelationship between these two phenomena.

Of interest is the finding that the delay in tissue reperfusion was not limited to the renal cortex but also occurred in the outer renal medulla of diabetic mice. Given the extensive generation of nitric oxide (NO) and other vasodilators such as bradykinin and PGE2 in the renal medulla (7, 25), one could expect that MBF would be better protected after ischemia than blood flow in the renal cortex. It is possible that the increase in MBF/CFR observed in the present study after renal ischemia may be related in part to the dominance of NO and other vasodilators in the renal medulla.

The contribution of NO to the regulation of blood flow in the diabetic kidney is complex (14). On the one hand, there is evidence that upregulation of the NO system, in particular of neuronal NO synthase and the endothelial NO synthase, may be involved in the induction of glomerular hyperfiltration in the early stages of diabetes (14, 15, 20, 30). However, additional studies in rats with streptozotocin-induced diabetes have demonstrated that there is a decline in the bioavailability of NO in the renal cortex (13, 26). Indeed, the study of Ishii et al. (13) suggested that such decreased availability of NO in diabetic animals could occur in a setting of a normal or increased NO production, due to inactivation of NO by superoxide anions. More recent studies in which cortical tubular fluid NO concentrations were measured directly, by a selective NO electrode, have suggested that species-related variations exist in different rodent models of early diabetes (19). Further studies, evaluating the magnitude of NO production and oxidative stress during renal ischemia in diabetic mice, as well as the activation inflammatory mediators and cell adhesion molecules, are required to determine the mechanisms that could contribute to the delayed renal reperfusion.

Because MAP was not assessed concomitantly with renal perfusion measurement, the possibility that changes in systemic blood pressure could contribute in part to the delay in renal reperfusion cannot be ruled out completely. However, a gradual decrease in MAP was noted in both db/db and db/m mice undergoing the same experimental procedure and anesthesia as in the original experiments, and there was no evidence for hemodynamic instability or deterioration in these mice. This suggests that the influence of MAP, if any, was minor and could not explain by itself the marked delay in renal reperfusion in the diabetic mice.

The findings of our study may have important clinical implications. In several recent reviews, diabetes mellitus was considered to be a predisposing factor for the development of ARF, among other situations such as preexisting renal impairment, cardiac disease, hypertension, jaundice, and advanced age (16–18). Patients with diabetes are known to be at a higher risk of developing ARF after administration of radiocontrast agents (32, 33). However, the question of whether patients with diabetes are more susceptible to ischemic injury and show a higher incidence of ischemic ARF is not entirely settled. Critically ill patients who are admitted to intensive care units are particularly prone to developing ischemic ARF. However, in a recent study aimed at identifying critically ill patients at a high risk for developing ARF, diabetes mellitus was not found to be a highly reliable predictor of ARF (6). Also, in a large series of patients with type 2 diabetes, most of the patients with

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**Table 1. Results of MAP measurements in surgically prepared db/db and db/m mice**

<table>
<thead>
<tr>
<th>Time and Procedure</th>
<th>db/db Mice, mmHg</th>
<th>db/m Mice, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline, after operation and before RA occlusion</td>
<td>86.0±19.0</td>
<td>77.5±11.3</td>
</tr>
<tr>
<td>During RA occlusion</td>
<td>78.5±12.6</td>
<td>70.5±15.0</td>
</tr>
<tr>
<td>After RA occlusion</td>
<td>72.6±10.2</td>
<td>73.0±8.3</td>
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Values are means ± SE. MAP, mean arterial pressure; RA, renal artery. Mice were anesthetized and prepared surgically in an identical manner to that performed during laser-Doppler measurements. MAP was measured by the tail-cuff technique.

levels are usually within the normal range. Therefore, in many studies, db/m mice serve as a control group to db/db mice. In our study, although the differences in several parameters between the db/m and db/db did not reach statistical significance, when the db/db mice and the “wild-type” C57BL/6J mice were compared, the difference became highly significant. It is therefore possible that some of the renal vascular disturbances are already present to a slight extent in the heterozygous state. Likewise, it is also possible that heterozygous mice are more prone to the development of hyperglycemia and other metabolic disturbances when exposed to stressful conditions like anesthesia. Indeed, their baseline blood glucose level was higher than that of C57BL/6J mice.

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acute or chronic renal failure had preexisting coronary heart disease, a history of administration of contrast media, or sepsis, suggesting a more complex etiology of ARF than pure ischemia (28). In view of the marked increase in the incidence of type 2 diabetes in recent years, validation of our findings in the clinical setting could be of importance to the identification of high-risk patients and could also have therapeutic implications.

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