Phosphodiesterase-5 inhibition attenuates early renal ischemia-reperfusion-induced acute kidney injury: assessment by quantitative measurement of urinary NGAL and KIM-1

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Acute hypoxia, toxic insult, or sepsis plays a role in renal ischemia-reperfusion (I/R) injury (3, 15, 29). Specifically, renal I/R injury is characterized by decreased oxygen delivery, depletion of cellular energy stores, and accumulation of toxic metabolites. The cellular depletion of ATP, a hallmark of ischemic injury, leads to a series of morphological, biochemical, and physiological derangements (16, 29, 31). Reperpfusion of ischemic tissue, although necessary for repair, has been shown to exacerbate acute ischemic injury owing to the generation of reactive oxygen and nitrogen species (2, 4, 16, 29, 31). The production of these agents initiates a cascade of events, including activation of mast cells and neutrophils and production of systemic inflammatory mediators and cytokines, which lead to death of renal cells via both apoptosis and necrosis (2, 4, 15, 16, 31).

Since I/R nephropathy poses an important clinical problem, several strategies have been proposed to deal with this disease (11, 16, 29). These include antioxidants and antioxidant enzyme mimetics, erythropoietin, peroxisome-proliferator-activated receptor agonists, inhibitors of poly(ADP-ribose) polymerase, carbon monoxide-releasing molecules, statins, adenosine, and acetylcysteine (4, 16, 29, 32). Unfortunately, the clinical efficacy of these therapeutic approaches is inconsistent and disappointing. Therefore, there is a great need for new, effective strategies to prevent kidney damage following I/R (11).

Phosphodiesterase-5 (PDE5) inhibitors were originally developed to treat angina pectoris and subsequently for erectile dysfunction and pulmonary hypertension (26). However, increasing evidence suggests that PDE5 inhibitors, including Sildenafil, Vardenafil, and Tadalafil, have broader effects, most likely due to their ability to inhibit the breakdown of cGMP, the second messenger for nitric oxide (NO) and natriuretic peptides (26, 28). Experimental studies have demonstrated that PDE5 inhibitors improve endothelial function (27) and reduce infarct size in rat models of myocardial infarction (23, 30). Hence, these inhibitors may also possess nephroprotective effects in renal I/R injury. Indeed, some studies showed that PDE5 inhibitors can exert beneficial renal effects in the I/R rat model (10), and following cardiopulmonary bypass AKI in swine (25). While these studies examined the effects of PDE5 inhibitor pretreatment on renal histology, oxidative stress, and function, the effects of PDE5 inhibitors on the more sensitive biomarkers of AKI (7, 8, 21, 22, 33), namely NGAL and KIM-1, have not been examined. Therefore, the present study was designed to determine whether Tada-
lafil can exert early nephroprotective effects in an I/R rat model, evaluated by kidney function, urinary NGAL, KIM-1, and renal histology.

MATERIALS AND METHODS

Studies were conducted on male Sprague-Dawley rats (Harlan Laboratories) weighing 300–350 g and maintained on standard rat chow (0.5% NaCl) and water ad libitum. The investigation was conducted according to the guidelines of the Animal Use and Care Committee, Technion. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, 1996) as approved by the local committee for supervision of animal experiments.

Experimental design. The animals were housed in air-conditioned vivarium under 12:12-h dark-light cycles and were given free access to rat chow and water for 2–3 days. Following an overnight fast, the animals were anesthetized with an Inactin anesthesia (100 mg/kg ip) and placed on a controlled heating (thermoregulated) table, keeping the body temperature at 37°C. Polyethylene tubes (PE50) were inserted into the right carotid artery for blood pressure monitoring and for blood draws, and the jugular vein for infusion of normal saline [0.9% NaCl/2% inulin/1% para-aminohippuric acid (PAH) at a rate of 3.0 ml/h (1% of body wt)], using syringe pumps. Arterial blood pressure was continuously monitored with a pressure transducer connected to the carotid arterial line. An additional catheter (PE50) was inserted into the bladder for urine collection after a suprapubicotomy cut. An upper abdominal midline incision was made and renal blood vessels were isolated bilaterally. A right nephrectomy was performed. After a bolus injection of 0.5 ml 1% inulin, followed by an equilibration period of 60 min, urine was collected into cooled tubes at predetermined times. After each urine collection, a reference blood sample was drawn. To minimize dehydration, the abdominal area was covered with saline-soaked gauze. After two constitutive baseline urine collections (30 min each), the left renal artery was clamped for 45 min followed by reperfusion for 60, 120, 180, and 240 min (group I: I/R + vehicle, n = 10). Animals of group II (n = 11) underwent the same procedure as in group I, with the exception that the rats were pretreated with Tadalafil (Lilly S. A. Alcobendas, Madrid, Spain; 10 mg/kg po via gavage) 24 h before renal ischemia and before the clamp (group II: I/R + Tadalafil). An equal amount of water (1 ml) was given to the rats in group I. Blood samples were centrifuged and serum was separated and stored along with urine samples at −20°C until further analysis. The applied dose of Tadalafil was chosen based on: 1) similar studies from the literature (9, 10) and 2) preliminary experiments from our lab, where a lower dose (5 mg·kg⁻¹·day⁻¹ po) was found to be less effective than the chosen dose in ameliorating renal dysfunction induced by I/R (data not shown). The 24-h pretreatment protocol was applied to explore the nephroprotective effect of PDE-I in I/R AKI model, in an attempt to mimic clinical situations were prophylactic potentially effective pretreatments are given for patients at high risk to develop AKI including those undergoing administration of radiocontrast or other nephrotoxic, cardiopulmonary bypass surgery, and renal artery clamping during partial nephrectomy or major vascular surgery.

At the end of the experiment, the left kidney was removed and sagitally sliced to two halves; one half of each kidney was stored in −70°C freezer and the other half was fixed in 10% neutral buffered formalin.

Histological evaluation. Hematoxylin and eosin staining was performed on paraffin-embedded 5-μm-thick longitudinal sections of kidneys. Morphological changes were assessed in a blinded fashion regarding treatment, and the extent of necrosis, retained casts, congestion, and inflammation was determined selectively in the cortical labyrinth, and in the outer and inner strips of the outer medulla. A semiquantitative analysis was performed, using a 0–4 score, with the 4th grade reflecting en block infarction of the entire region examined, casts detected in all tubuli, extensive congestion and extravasation of red cells into the parenchyma, and extensive polymorphonuclear infiltration, respectively, as described by Kiris et al. (14). Kidneys from normal untreated rats served as controls.

Biochemical analyses. Sodium concentration in plasma and urine was determined by flame photometry (model IL 943, Instrumentation Laboratories, Milan, Italy). Inulin and PAH concentrations were measured by the colorimetric method. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were equated with the clearance of inulin and PAH, respectively. Urinary NGAL and KIM-1 levels were measured using commercially available ELISA kits (Bio Porto, Gentofte, Denmark) and (Wuhan ELAab Science, Wuhan, China), respectively.

Statistical analysis. Statistical significance was assessed by one-way ANOVA for repeated-measures, or two-way ANOVA, as appropriate. The Dunnett test and Tukey’s multiple comparisons test were used for data point comparisons in each group. P ≤ 0.05 was considered statistically significant. Data are presented as means ± SE.

RESULTS

Effects of Tadalafil on renal hemodynamics and clearance parameters in rats with I/R. The effects of Tadalafil on kidney excretory functions and mean arterial pressure (MAP) in rats with ischemic AKI are summarized in Fig. 1. In line with the renal excretory effects of PDE5 inhibitors, baseline values of urinary flow (V), urinary Na⁺ excretion (UNaV), and fractional Na⁺ excretion (FENa) tended to be higher in rats pretreated with Tadalafil compared with vehicle-treated rats (Fig. 1, A, B, C). Baseline MAP was slightly lower in rats treated with Tadalafil compared with untreated animals (Fig. 1, D). Nevertheless, none of the differences in the excretory (V and UNaV) or hemodynamic (MAP) baseline values between the two groups was statistically significant. As expected, in-

![Fig. 1. Effects of Tadalafil on urinary flow (V; A), urinary Na⁺ excretion (UNaV; B), fractional Na⁺ excretion (FENa; C), and mean arterial pressure (MAP; D) in rats with acute kidney injury (AKI) induced by renal ischemia-reperfusion (I/R). *P < 0.05 compared with baseline value. #P < 0.05 compared with untreated I/R AKI group. Data are expressed as means ± SE.](http://ajprenal.physiology.org/Download.pdf)
duction of ischemic AKI in untreated rats provoked gradual and significant increases in V, UNaV, and FENa, which persisted throughout the followup period (Fig. 1). When the same ischemic renal insult was induced in rats pretreated with Tadalafil, the increases in these parameters were significantly lower than those observed in untreated animals (Fig. 1).

The changes in GFR and RPF following renal I/R in rats, with or without Tadalafil treatment, are depicted in Fig. 2. Untreated rats that were exposed to I/R exhibited gradual and significant decline in GFR, but not of RPF (Fig. 2). Tadalafil-pretreated rats displayed reduced baseline values of GFR (P = 0.05) and RPF compared with untreated animals. This adverse effect may be secondary to the hypotensive action of this PDE inhibitor (PDE-I). When renal I/R was induced in Tadalafil-pretreated animals, the decline in GFR was much milder than that observed in untreated animals. While Tadalafil-pretreated rats recovered kidney function after 4 h, untreated I/R animals continued to display hypofiltration at this time point.

Effects of Tadalafil on urinary excretion of NGAL and KIM-1 in rats with I/R. The effects of Tadalafil on urinary excretion of NGAL and KIM-1 in rats with AKI are summarized in Fig. 3. In the untreated I/R group, urinary excretion of NGAL increased gradually from baseline levels of 9.6 ± 2.8 to a maximal value of 271.9 ± 34.3 ng/min (P < 0.05), 4 h after the induction of the renal injury (Fig. 3A). In rats that underwent I/R and were pretreated with Tadalafil, the increase in urinary NGAL was significantly attenuated; it increased from 6.72 ± 1.6 to 174.9 ± 22.4 ng/min (P < 0.05). This beneficial effect of Tadalafil was evident 1 h after I/R and lasted throughout the experiment.

As can be seen in Fig. 3B, a similar trend in urinary excretion of KIM-1 was obtained. Specifically, I/R was associated with a gradual increase in urinary KIM-1 from baseline levels of 2.71 ± 0.67 to maximal levels of 182.6 ± 77.08 ng/min (P < 0.05), 3 h after renal insult, and substantial decline thereafter. In contrast, urinary NGAL continued to be elevated 4 h after I/R. Noteworthy, Tadalafil pretreatment prevented the rise in urinary KIM-1 after I/R.

Histological analysis. The nephroprotection conferred by pretreatment with Tadalafil before I/R was also supported by histological analysis of renal tissue of the various experimental groups.

Figure 4 shows representative images of renal histological slides from normal rats (top lane), rats that underwent I/R alone (middle lane), and rats that underwent I/R after treatment with Tadalafil (bottom lane). Light microscopy 4 h after I/R revealed marked changes in renal histology, including tubular dilatation or collapse, loss of the brush border, and cellular detachment from tubular basement membranes. Necrosis and cast formation were most prominent in the outer stripe, whereas congestion was more intense in the inner stripe. Papillary changes were minimal (not shown) and polymorphonuclear infiltration was not evident at this stage. Consistent with the functional data, all these lesions were substantially reduced in rats exposed to Tadalafil (Fig. 4 and Fig. 5).

DISCUSSION

The present study demonstrates that pretreatment (prophylactic) with oral Tadalafil, a PDE5 inhibitor, exerts renopro-
Tadalafil inhibition may constitute a potential renoprotective approach against ischemic AKI induced by renal artery clamping. The nephroprotective effects of Tadalafil were evident at the functional level as well as at the cellular level. Specifically, Tadalafil completely ameliorates kidney hypofiltration 4 h after the I/R. In addition to the improvement in renal function, Tadalafil substantially reduced the histological alterations induced by I/R including tubular damage, cellular detachment, cast formation, congestion, and necrosis. Likewise, the expected increases in sensitive biomarkers of renal injury such as NGAL and KIM-1 were largely modified by this agent.

AKI due to I/R injury may be caused by various etiologies ranging from hypoperfusion, which is common in vascular and cardiac surgery, to the more severe form, namely, intrinsic AKI. While prerenal azotemia is characterized by normal kidney histology, the intrinsic severe form of ischemic AKI is associated with a series of morphological, biochemical, and physiological derangements (16, 29, 31). These may include vascular and tubular injury evident by cellular detachment, cast formation, congestion, necrosis, and increased production of injury markers such as NGAL and KIM-1 (12, 13, 20). Although reperfusion of ischemic tissue is necessary for its recovery, it may aggravate the acute ischemic injury through the generation of reactive oxygen and inflammation, leading to death of renal cells by apoptosis and necrosis (2, 4, 15, 16, 29, 31). It is well-known that the production of free oxygen radicals takes place within the first few minutes of reperfusion, thus reperfusion injury is profound during the early period (13). Regardless of the etiology, AKI negatively affects prognosis of patients. Therefore, several maneuvers, including anti-oxidants, have been applied to reduce the susceptibility of the renal tissue to I/R damage. Disappointingly, the therapeutic efficacy of these approaches has been inconsistent, ranging from absence of nephroprotective effects to partial beneficial effects (19). The present study shows that early PDE5 inhibition may be a potential therapeutic prophylactic approach in preventing kidney dysfunction and reduction of structural renal damage associated with I/R AKI. This notion is further supported by our findings that Tadalafil markedly reduced urinary excretion of KIM-1 and NGAL following I/R rats. These findings are consistent with the results of Guzeloglu et al. (10) who demonstrated that Tadalafil exerted renal beneficial effects in an I/R rat model. However, these authors examined the effects of Tadalafil pretreatment (60 min before I/R) on histological changes and oxidative stress in a model of 60 min of ischemia and 60 min of reperfusion without evaluation of kidney function. Similarly, Sildenafil, a short acting member of the PDE5 inhibitor family, was effective in preventing kidney dysfunction and preserving NO bioavailability in an I/R AKI model in rats (6, 18, 24) and in an experimental postcardiopulmonary bypass-induced AKI (25). The nephroprotective effects of Sildenafil are not restricted to I/R AKI, rather they are also evident in other AKI models such as cisplatin-induced nephrotoxicity (17). In contrast to these findings, Faddegan et al. (9) reported that pretreatment with Tadalafil improves preoperative renal function, but it does not mitigate renal damage and kidney dysfunction in porcine I/R AKI model. None of these studies examined whether the nephroprotective effects of PDE5 inhibitors are associated with reduced urinary excretion of sensitive and specific kidney injury novel biomarkers. Thus, our findings that pretreatment with Tadalafil substantially reduced the urinary excretion of KIM-1 and NGAL following I/R rats. These findings are consistent with the results of Guzeloglu et al. (10) who demonstrated that Tadalafil exerted renal beneficial effects in an I/R rat model. However, these authors examined the effects of Tadalafil pretreatment (60 min before I/R) on histological changes and oxidative stress in a model of 60 min of ischemia and 60 min of reperfusion without evaluation of kidney function. Similarly, Sildenafil, a short acting member of the PDE5 inhibitor family, was effective in preventing kidney dysfunction and preserving NO bioavailability in an I/R AKI model in rats (6, 18, 24) and in an experimental postcardiopulmonary bypass-induced AKI (25). The nephroprotective effects of Sildenafil are not restricted to I/R AKI, rather they are also evident in other AKI models such as cisplatin-induced nephrotoxicity (17). In contrast to these findings, Faddegan et al. (9) reported that pretreatment with Tadalafil improves preoperative renal function, but it does not mitigate renal damage and kidney dysfunction in porcine I/R AKI model. None of these studies examined whether the nephroprotective effects of PDE5 inhibitors are associated with reduced urinary excretion of sensitive and specific kidney injury novel biomarkers. Thus, our findings that pretreatment with Tadalafil reduced the excretion of NGAL and KIM-1 in I/R AKI extend current knowledge and provide novel evidence that PDE5 inhibition may constitute a potential renoprotective approach against ischemic AKI.

The mechanisms underlying the nephroprotective effects of Tadalafil in I/R are not fully understood. Tadalafil, as other PDE5 inhibitors, prevents the breakdown of NO- and NP-derived cGMP, primarily in vascular smooth muscle cells, thus inducing vasodilator effects (26, 28). At the renal level, PDE5 is localized to the vasculature, glomeruli, mesangial cells,
cortical tubules, and inner medullary collecting duct cells of rat kidney, where its inhibition positively affects renal hemodynamic and excretory function (10, 23, 25–28, 30). The vasodilatory action of Tadalafil is of a special importance in light of the intrarenal activation of vasoconstrictory systems (endothelin, adenosine, and angiotensin II) that contribute to reduction in GFR, together with vascular congestion in the outer medulla and activation of tubuloglomerular feedback. On the other hand, we and others showed that renal I/R injury is characterized by downregulation of endothelial NO synthase (eNOS) and upregulation of inducible NOS (iNOS) (1, 17). While eNOS plays a pivotal protective role in I/R kidney injury, iNOS adversely affects renal function/structure during I/R in the kidney (1). Using a similar AKI model, Choi et al. (6) demonstrated that Sildenafil significantly enhanced iNOS and eNOS in the renal tissue of I/R rats compared with the vehicle-treated animals. However, in the current study, we did not observe any changes in RBF following Tadalafil treatment despite the increase in GFR, suggesting that other pathways affecting filtration fraction may exist. For instance, the decline in cast formation, necrosis, and congestion might have contributed to the observed renoprotective effects of Tadalafil in I/R. Apoptosis and necrosis are common features following renal I/R injury (3). In this context, Choi et al. (6) demonstrated that apoptosis was associated with upregulation of proapoptotic protein (Bax), downregulation of antiapoptotic protein (Bcl-2), and increased caspase-3 activity in the renal tissue. Noteworthy, Sildenafil pretreatment significantly increased Bcl-2 and decreased Bax and cleaved caspase-3 following I/R in rats compared with vehicle-treated animals, demonstrating the antiapoptotic capacity of Sildenafil in renal I/R injury. Furthermore, the number of TdT-mediated dUTP nick end labeling-positive cells was significantly lower in the kidneys from Sildenafil-treated IR rats compared with the vehicle-treated IR rats (6).

In sum, this study demonstrates that Tadalafil pretreatment modified the recovery of renal injury induced by I/R in experimental rats. This beneficial effect was evident by attenuating the decline in renal function as well as the deleterious renal histological changes such as cast formation, congestion, and necrosis.

Fig. 5. Quantification of cast formation, congestion, and necrosis in normal rats, I/R group, and I/R + Tadalafil group. *P < 0.05, **P < 0.01 vs. normal rats. #P < 0.05 vs. untreated I/R group.
Moreover, Tadalafil significantly reduced the urinary excretion of NGAL and KIM-1, two sensitive biomarkers of kidney injury. The ability of Tadalafil to induce ischemic tolerance suggests that this drug may be used as a potential prophylactic agent in clinical settings characterized by ischemic AKI such as kidney transplantation, partial nephrectomy, cardiopulmonary bypass, and radiocontrast administration. Additional studies are needed to explore whether Tadalafil is nephroprotective also when given as a late treatment in clinical and experimental AKI.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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