Promising effects of ischemic preconditioning in renal transplantation

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Promising effects of ischemic preconditioning in renal transplantation.

Background. Ischemic preconditioning, a phenomenon induced by brief ischemia and reperfusion periods, renders an organ tolerant to subsequent prolonged ischemia. This study evaluated different schedules of preconditioning the kidney to assess the role of nitric oxide (NO) and determine the effects of preconditioning on kidney transplantation.

Methods. In study design A, to determine the optimum procedure of preconditioning, one-cycle schedules were assayed by occluding/releasing renal pedicles according to various warm ischemic (5, 10, 15, 20 min) and reperfusion (10, 20, 40 min) windows in Sprague-Dawley rats. Thereafter, warm renal ischemia was induced by clamping both pedicles for 40 minutes. Design B used the most suitable schedule found from the first study to obtain several groups, using either a direct nitric oxide donor (spermine NONOate) or two nitric oxide synthase (NOS) blockers (L-NAME and aminoguanidine), to determine whether NO mediates in renal preconditioning. To establish whether preconditioning reduces cold preservation damage, in Design C the optimum preconditioning schedule was used in syngeneic Lewis rats where preconditioned and non-preconditioned kidneys were transplanted after five hours of cold storage in Euro-Collins solution.

Results. The best preconditioning schedule consisted of 15 minutes of warm ischemia and 10 minutes of reperfusion (Prec 15/10), since it was the only schedule that offered both functional and histological protection. The NO donor reproduced the ischemic preconditioning. Non-selective NOS blockade abolished the preconditioning and exacerbated ischemic damage, which was overcome by the addition of the NO donor. Selective blocking of inducible NOS also abolished the effects of preconditioning. Renal NO increased at the end of preconditioning in the Prec 15/10 group. Prolongation of the reperfusion window (20 or 40 min) abolished the preconditioning protection, although it was associated with a further increase in renal NO. As renal DNA oxidative injury paralleled NO, increasing with prolongation of reperfusion, it may account for the disappearance of preconditioning. Finally, the one-cycle preconditioning schedule offered an effective functional and histological protection against cold preservation damage in rat renal transplantation.

Conclusions. Fifteen minutes of warm ischemia and 10 minutes of reperfusion in the kidney is the most suitable one-cycle schedule for preconditioning since it protects from both warm and cold ischemia. The beneficial effect of preconditioning is related to the local production of NO, and we believe it has promising therapeutic value in clinical renal transplantation.

In renal transplantation, the deleterious effect of delayed graft function secondary to ischemia-reperfusion damage may act to trigger or amplify host allo-response and influence both the acute rejection occurrence [1] and late chronic changes [2]. Thus, protective maneuvers at the time of transplantation should clearly benefit the fate of the organ.

Ischemic preconditioning, a phenomenon induced by brief ischemia and reperfusion periods, renders an organ more tolerant to subsequent sustained ischemia-reperfusion [3]. This phenomenon has been mainly studied and characterized in the heart [3, 4], but it has also been described in the liver [4–6], the small intestine [7, 8] and the brain [9]; however, little information available in the kidney. A report in the early 1980s focused on the late acquisition of resistance against ischemic injury through the induction of intrinsic antioxidant enzymes by a previous episode of short ischemia [10]. This is a protein-dependent mechanism similar to the late phase of preconditioning described in the heart [11]. More recently, some studies concerning early protection of renal tissue by ischemic preconditioning have been drafted [12–14] with contradictory results. All these studies used a four-cycle preconditioning schedule similar to that classically applied in the heart [11, 15]. Using an easier one-cycle schedule, preconditioning has been achieved in some

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organ [6, 8] and our preliminary experiment ensured protection of the kidney against warm ischemia [16].

Although the mechanisms of ischemic preconditioning are unclear, potential mediators include heat shock proteins [17, 18], endothelin [19], nitric oxide (NO) [15, 20] and adenosine [5, 6, 9, 14]. The production of NO affects organ integrity in response to various challenges. In this sense, l-arginine and NO donors provide significant protection in ischemia-reperfusion in several organs [21, 22], while exogenous NO administration may also mimic ischemic preconditioning [23]. In addition, inhibition of NO can induce or aggravate most of the alterations elicited by ischemic damage [24, 25]. In the kidney, NO is involved in ischemia-reperfusion injury [22, 26], but few data are available regarding its role in ischemic preconditioning.

Thus, we first aimed to assess whether preconditioning can be induced in the kidney and if so, which is the optimum schedule of preconditioning. To discover the mechanisms of preconditioning, we also studied the role of NO in kidney preconditioning by treating with NO donors and by inhibiting nitric oxide synthase (NOS). Finally, since the major interest of renal ischemic preconditioning should be its applicability to clinical transplantation, we determined whether this protective effect could be extended to kidney preservation.

METHODS

Animals and surgical procedure

All procedures followed the Guidelines of the European Community Committee on Care and Use of Laboratory Animals and Good Laboratory Practice. Warm ischemia studies were carried out in male Sprague-Dawley rats (250 to 300 g body wt) from our in-house breeding facility. Under intramuscular (IM) anesthesia with a mixture of ketamine (75 mg/kg), atropine (0.05 mg/kg) and diazepam (5 mg/kg), a midline laparotomy was performed, renal vessels were dissected, and warm ischemic damage was induced by cross-clamping both renal pedicles for 40 minutes. During ischemia, animals were kept at 37°C. Preliminary data from our laboratory indicated that cross-clamping of both the renal artery and vein for 40 minutes produced consistent ischemic injury.

Cold ischemia studies were carried out following a renal transplantation (Tx) model, in which donors and recipients were inbred male Lewis rats (250 g body weight; Charles River, Sta. Perpetua de la Mogoda, Barcelona, Spain) and surgery was performed as described elsewhere [27, 28]. For kidney harvesting and transplantation, anesthesia was induced and maintained by an IM injection of Droperidol (neuroleptoanalgesic; 0.5 mg/100 g body weight) + Fentanyl (analgesic; 0.01 mg/100 g body weight). For left kidney harvesting, a single dose of sodium heparin (1000 IU) was administered and the kidney was immediately washed with Euro-Collins solution (EC; 4 mL, 4°C, 20 mL/h flow rate). Thereafter, the kidney was either transplanted immediately or preserved in EC at 4°C before transplantation. For heterotopic renal transplantation, the host left kidney was nephrectomized, the donor artery and vein were anastomosed end-to-side to the receptor aorta and cava, and the ureter was anastomosed end-to-end. Re-anastomosis took no more than 30 minutes and total surgical time did not exceed 60 minutes. The grafts that did not reperfuse correctly were rejected.

After surgery, animals were housed in a room kept at constant temperature with a 12 h/12 h light/dark cycle. Rats were fed a standard diet and tap water ad libitum. Before surgery and on days 1, 2, 3 and 7, animals were weighed and a blood sample was obtained from the tail vein for serum creatinine (S_Cr) measurement (µmol/L), which was performed by an autoanalyzer (Beckman Instruments, Palo Alto, CA, USA) following Jaffe’s reaction. Rats were killed under ketamine anesthesia, blood was obtained by aortic puncture and the kidneys were processed for tissue studies.

Histological studies

For conventional histology, coronal 1- to 2-mm thick slices of the kidney were fixed in 4% formaldehyde and embedded in paraffin, and 3- to 4-µm thick tissue sections were stained with hematoxylin and eosin (H&E), and by the periodic acid-Schiff (PAS) methods. Light microscopy sections were examined by a pathologist who was blinded to the treatment groups. Samples were analyzed for tubular cell necrosis, tubular dilation, intratubular cell detachment, interstitial edema and interstitial cellular infiltrate. Abnormalities were graded using a semiquantitative scale from 0 to 4+, in which 0 denotes no abnormalities, 1+ = changes affecting <25% of the sample, 2+ = changes affecting 25 to 50% of the sample, 3+ = changes affecting 50 to 75% of the sample and 4+ = changes affecting >75% of the sample.

Study design

Design A. To study the effect of ischemic preconditioning on warm ischemia and obtain the most suitable preconditioning schedule, two sets of experiments were performed. On one hand, four warm ischemic preconditioning times (5, 10, 15 and 20 min) with a constant reperfusion time of 10 minutes prior to the 40 minutes of warm ischemia were analyzed. For this purpose, forty animals were classified in five groups: (1) the ISC group had no preconditioning and 40 minutes of warm ischemia (N = 8); (2) the Prec 5/10 group had 5 minutes of warm ischemia, 10 minutes of reperfusion, and 40 minutes of warm ischemia (N = 8); (3) the Prec 10/10 group had 10 minutes of warm ischemia, 10 minutes of reperfusion, and 40 minutes of warm ischemia (N = 8); (4) the Prec
15/10 group had 15 minutes of warm ischemia, 10 minutes of reperfusion, and 40 minutes of warm ischemia (N = 8); and (5) the Prec 20/10 group had 20 minutes of warm ischemia, 10 minutes of reperfusion, and 40 minutes of warm ischemia (N = 8).

Secondly, given the optimum effect of 15 minutes of warm ischemic preconditioning, we tested the effects of increasing the reperfusion time (20 and 40 min). To this end, twelve animals were organized into two other groups: (1) the Prec 15/20 group had 15 minutes of warm ischemia, 20 minutes of reperfusion, and 40 minutes of warm ischemia (N = 6); and (2) the Prec 15/40 group had 15 minutes of warm ischemia, 40 minutes of reperfusion, and 40 minutes of warm ischemia (N = 6).

**Design B.** Using the best schedule of preconditioning from Design A, that is, 15 minutes of warm ischemia followed by 10 minutes of reperfusion (Prec 15/10; hereafter named IP), we determined whether NO could mediate ischemic preconditioning. For this purpose, another set of experiments was performed that included the following groups: (1) ISC group with 40 minutes of warm ischemia (N = 9); (2) IP-ISC group, which had IP plus 40 minutes of warm ischemia (N = 14); and (3) NO-ISC group administered spermine NONOate (10 mg/kg) IV 5 minutes before ischemia, and then 40 minutes of warm ischemia (N = 12).

Additional groups were studied to analyze the effects of blocking NOS on preconditioning: (1) NA-IP group, where the non-selective blocker L-NAME (10 mg/kg) was added IV 5 minutes before surgery, then IP and finally 40 minutes of warm ischemia (N = 14); (2) NO-NA-IP group had spermine NONOate (10 mg/kg) and the non-selective blocker L-NAME (10 mg/kg) administered IV 5 min before surgery, IP, and 40 minutes of warm ischemia (N = 9); (3) NA-ISC, where the non-selective blocker L-NAME (10 mg/kg) was added IV 5 minutes before surgery, and then 40 minutes of warm ischemia (N = 6); (4) AG-IP group, the selective inducible NOS blocker aminoguanidine (150 mg/kg) administered subcutaneously (SC) one hour before surgery, IP, and 40 minutes of warm ischemia (N = 9); and (5) AG-ISC group, the selective inducible NOS blocker aminoguanidine (150 mg/kg) added SC one hour before surgery, 40 minutes of warm ischemia (N = 6). The renal function in this set of experiments was monitored for only three days and no histological evaluation was performed.

Finally, more animals were added to the Prec 15/10 (N = 8), Prec 15/20 (N = 10), Prec 15/40 (N = 10), NA-IP (N = 10) and AG-IP (N = 10) groups to assess tissue NO and oxidized DNA. These animals followed the same preconditioning schedule as described above except that the tissue was freeze-clamped instead of applying 40 minutes of warm ischemia. Kidneys from a Sham (N = 7) group were added.

**Design C.** Finally, to determine whether preconditioning affected cold ischemic damage, 21 rats were classified into three groups: (1) NoCOLD group, where kidneys were flushed with EC solution at 4°C and immediately transplanted (N = 7); (2) COLD5H group, where kidneys were flushed with EC at 4°C and stored for 5 hours before transplantation (N = 7); and (3) IP + COLD5H group, where after IP, the kidneys were flushed with EC at 4°C and then stored for 5 hours before transplantation (N = 7).

**Tissue nitrite and nitrate measurements**

Nitric oxide production in renal tissue was determined by tissue accumulation of nitrite and nitrate as described elsewhere [29]. Briefly, frozen kidney sections were weighed and homogenized in 2 mL of 100 mmol/L Tris-HCl, pH 7.4 at 4°C. Proteins were precipitated in 1 mL of homogenate by adding 200 µL of 1 N HCl. After centrifugation, the supernatant was adjusted to pH 7.6 with 100 µL of 1 N NaOH plus 300 µL of 100 mmol/L Tris-HCl. Total NO products were measured in the supernatant with a commercial kit from Cayman Chemical (Ann Arbor, MI, USA). Nitrate was reduced to nitrite by a three-hour incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH) and flavin-adenine-dinucleotide. Nitrite was converted into a deep purple azo compound by the addition of Griess reagent. Photometric measurement of the absorbance at 540 nm provided nitrite concentration. Protein concentration was determined in the other milliliter of homogenate following Bradford, with bovine serum albumin (BSA) as a standard. Results were expressed as µmol NO/mg protein.

**Detection in situ and evaluation of DNA oxidation**

To assess the involvement of free radical formation in ischemia-reperfusion, we identified 8-oxo-2’-deoxyguanosine, a sensitive marker of DNA base damage, by a modification of the Biotrin OxyDNA assay (BIOTRIN Int Ltd, Dublin, Ireland). This test is based on an affinity technique that allows direct identification of DNA damage in pathological specimens and in cultures in vitro [30]. The OxyDNA assay applies a direct fluorescent technique for the detection of oxidative damage to DNA. Briefly, 5-µm thick tissue sections were pretreated with proteinase K (20 mg/mL) for 15 minutes. Then, the FITC conjugated with a specific oxyDNA probe, which binds to 8-oxo-2’-deoxyguanosine in damaged cells, was added. All sections were examined under fluorescence microscope and semiquantitatively graded on a scale from 0 to 3+, where 0 denotes no fluorescence, and 1, 2 and 3 mild, moderate and strong fluorescence, respectively.

**Statistical analysis**

The Chi square test was used to compare the mortality ratio from acute renal failure between treated and non-
treated groups. To compare more than two groups for proteinuria and serum creatinine throughout the follow-up, a one-way analysis of variance (ANOVA) followed by Scheffe’s test was performed. To compare histological data, the non-parametric Kruskal-Wallis test and subsequent Conover’s test were applied. All P values were two-tailed, and a P value of less than 0.05 was considered statistically significant. Data are the mean ± standard error.

RESULTS
Protective effect of preconditioning on warm ischemia to establish the optimal one-cycle preconditioning schedules

The serum creatinine ($S_{Cr}$) profile is shown in Figure 1A. Rats from the ISC group presented severe renal failure on the first day and serum creatinine worsened until a peak on the second day. On the seventh day these animals showed a $S_{Cr}$ concentration higher than the baseline value. On the first day, serum creatinine in the Prec 5/10, Prec 10/10 and Prec 15/10 groups was significantly lower than in ISC rats. In contrast, the 20/10 preconditioning schedule did not protect against ischemia since the $S_{Cr}$ profile in the Prec 20/10 group was similar to that in ISC rats. All ischemic preconditioning groups showed progressive amelioration of renal function on the second and third days after ischemia. Serum creatinine levels in the Prec 5/10, Prec 10/10 and Prec 15/10 groups returned to basal values, whereas that of the Prec 20/10 did not. Among preconditioning groups, rats with 15 minutes of ischemic preconditioning (Prec 15/10) had the best renal function on the first and second days after ischemia.

Histological evaluation revealed severe tubulointerstitial damage in kidneys from the ISC group, while only the Prec 15/10 group had a significantly lower degree of histological damage, as evidenced by lower tubular necrosis, medullar congestion and hemorrhage, and development of proteinaceous casts (Table 1). The other preconditioning groups showed tubulointerstitial damage similar to that of ISC group.

In the groups with the reperfusion window prolonged to 20 or 40 minutes (Prec 15/20 and Prec 15/40 groups) the $S_{Cr}$ was higher than in Prec 15/10 animals from the first day. This difference reached significance on the second and third days (Fig. 1B). Tubulointerstitial damage in these groups was clearly stronger than in Prec 15/10 animals and similar to that of the ISC group (Table 1).
Effect of NO donor on warm ischemia and effect of NOS inhibition on preconditioning

Nitric oxide addition with a direct NO donor to animals subjected to 40 minutes of warm ischemia decreased $S_\text{Cr}$ to levels even lower than in preconditioned animals (Table 2). This indicates that NO donors provide significant protection against dysfunction resulting from warm renal ischemia.

When NOS was inhibited non-selectively with L-NAME, the effect of preconditioning was abolished, and severe renal failure was observed with $S_\text{Cr}$ notably higher than in ISC animals (Table 2). Furthermore, 6 of 14 animals (43%) from this NA-IP group died because of uremia, suggesting that the ischemic damage was also greater. Accordingly, in the group of non-preconditioned animals receiving L-NAME (NA-ISC group), comparable high mortality rates (33%, 2 of 6) and severe renal failure were observed (Table 2). When we added the NO donor to the group of preconditioned animals receiving L-NAME (NO-NA-IP group), $S_\text{Cr}$ fell to levels similar to those found in preconditioned animals.

Finally, selective blocking of inducible NOS with aminoguanidine in preconditioned animals (AG-IP group) abolished ischemic preconditioning and $S_\text{Cr}$ increased to levels similar to those in ISC animals. Accordingly, the group of non-preconditioned animals receiving aminoguanidine (AG-ISC group) presented a $S_\text{Cr}$ profile similar to that of ISC animals. Neither group showed mortality.

Nitric oxide content in preconditioned tissue

Tissue NO, as measured by nitrate and nitrate levels, was significantly higher after 15 minutes of warm ischemia and 10 minutes of reperfusion, the preconditioning 15/10 window, than in sham-operated rats (Fig. 2). When reperfusion was prolonged to 20 and 40 minutes, nitrite and nitrate levels increased significantly. Both the non-selective and inducible NOS blocking decreased tissue NO after the preconditioning 15/10 window, to a value similar to that found in Sham animals.

Detection in situ and evaluation of DNA oxidation

The extent of DNA oxidation, as measured by tissue 8-oxo-2′-deoxyguanosine, showed a profile parallel to that of nitric oxide with a higher fluorescence intensity in the group of the preconditioning 15/10 window than in sham-operated rats (Figs. 2 and 3). As reperfusion was prolonged, both the intensity and number of positive cells increased, suggesting higher DNA oxidation by oxygen free radicals.

Protective effect of preconditioning on cold ischemia

All animals survived the study period. Over the seven-day follow-up, $S_\text{Cr}$ in NoCOLD group was similar to pre-operative values. Animals grafted after five hours of cold ischemia (COLD5H group) had significantly higher $S_\text{Cr}$ levels than NoCOLD animals throughout the study. On the first day, $S_\text{Cr}$ in IP + COLD5H increased as in the COLD5H group. However, on the following days it steadily decreased to values significantly lower than in COLD5H and similar to those found in NoCOLD animals (Fig. 4).

The histological evaluation was consistent with functional results. The non-ischemic group (NoCOLD) had scarcely appreciable lesions, whereas the COLD5H group displayed moderate-severe ischemic-characteristic tubulointerstitial lesions. The IP + COLD5H group showed some ischemic tubulointerstitial abnormalities, which were clearly slighter than in the COLD5H group (Table 3 and Fig. 5).

DISCUSSION

Preconditioning is a simple and harmless method used to render an organ tolerant to ischemia. Here, we examined whether a one-cycle schedule of preconditioning attenuated the damage induced by sustained ischemia in the kidney and then determined the optimal time window of ischemia and reperfusion. The main finding is that a transient and short episode of warm ischemia and reperfusion protects the kidney from subsequent warm or cold ischemia insults by a mechanism in which NO is involved.

In the warm ischemia setting, one-cycle of 15 minutes of ischemia followed by 10 minutes of reperfusion was the optimal time schedule, as it prevented both renal function and morphology. Alternatively, we showed that either 5 or 10 minutes of ischemia followed by 10 minutes of reperfusion offered functional but partial structural protection. However, when the ischemic window was prolonged beyond 15 minutes, the preconditioning effect disappeared. Previous studies on warm renal ischemia [12, 13] used a four-cycle preconditioning schedule, which
Table 2. Effects of nitric oxide (NO) donor and NO synthase (NOS) inhibition on ischemic preconditioning

<table>
<thead>
<tr>
<th>Prec 15-10</th>
<th>NO donor</th>
<th>NOS inhibitor</th>
<th>S0 0</th>
<th>S0 1</th>
<th>S0 2</th>
<th>S0 3</th>
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<tr>
<td>ISC</td>
<td>—</td>
<td>—</td>
<td>45 ± 2</td>
<td>273 ± 20bc</td>
<td>274 ± 43bc</td>
<td>228 ± 49bc</td>
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<tr>
<td>IP-ISC</td>
<td>Yes</td>
<td>—</td>
<td>46 ± 2</td>
<td>182 ± 25</td>
<td>121 ± 20</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>NO-ISC</td>
<td>Yes</td>
<td>—</td>
<td>44 ± 3</td>
<td>103 ± 11bc</td>
<td>74 ± 4</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>NA-ISC</td>
<td>—</td>
<td>L-NAME</td>
<td>46 ± 2</td>
<td>268 ± 10bc</td>
<td>227 ± 28bc</td>
<td>157 ± 3bc</td>
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<tr>
<td>NA-IP</td>
<td>Yes</td>
<td>—</td>
<td>45 ± 1</td>
<td>327 ± 14bc</td>
<td>409 ± 43bcd</td>
<td>289 ± 49bc</td>
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<tr>
<td>NO-NA-IP</td>
<td>Yes</td>
<td>Yes</td>
<td>45 ± 2</td>
<td>191 ± 32</td>
<td>122 ± 24</td>
<td>86 ± 15</td>
</tr>
<tr>
<td>AG-ISC</td>
<td>—</td>
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<td>46 ± 2</td>
<td>263 ± 21bc</td>
<td>318 ± 44bc</td>
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<tr>
<td>AG-IP</td>
<td>Yes</td>
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<td>44 ± 2</td>
<td>295 ± 17bc</td>
<td>295 ± 52bc</td>
<td>221 ± 40bc</td>
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<td>—</td>
<td>0.0001</td>
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Using the 15/10 preconditioning schedule, several groups were studied using either a direct NO donor, spermine NONOate (NO, 10 mg/kg), or two NOS blockers (L-NAME 10 mg/kg and aminoguanidine, AG 150 mg/kg). Definitions of groups are: ISC group, 40 min of warm ischemia; IP-ISC group, preconditioning 15/10, 40 min of warm ischemia; NO-ISC group, spermine NONOate, 40 min of warm ischemia; NA-IP group, the non-selective blocker L-NAME, preconditioning 15/10, 40 min of warm ischemia; NO-NA-IP group, spermine NONOate, L-NAME, preconditioning 15/10, 40 min of warm ischemia; AG-IP group, the selective inducible NOS blocker aminoguanidine, preconditioning 15/10, 40 min of warm ischemia; and AG-ISC group, aminoguanidine, 40 min of warm ischemia. In the NA-IP and NA-ISC groups, 43 and 33% mortality was observed, respectively, because of uremia. Statistical analysis is by ANOVA/Scheffe’s test.

$^aP < 0.05$ vs. IP
$^bP < 0.05$ vs. NO-ISC
$^cP < 0.05$ vs. NO-NA-IP
$^dP < 0.05$ vs. ISC, AG-ISC and AG-IP

Fig. 2. Tissue nitric oxide and DNA oxidation after one cycle preconditioning. (A) Nitric oxide (NO) production in renal tissue was determined by tissue accumulation of nitrite and nitrate. DNA oxidative damage was evaluated by a direct fluorescent technique, identifying 8-oxo-2'-deoxyguanosine. (B) All sections were examined with fluorescence microscopy and semiquantitatively graded from 0 to 3, where 0 represents no fluorescence intensity, and 1, 2, 3, mild, moderate and strong fluorescence intensity respectively. Statistical analysis was by the Kruskal-Wallis/Connover test; * $P < 0.05$.

is the most widespread method used in experimental models [11, 15]. However, this procedure may be confusing and complex, and results have not always been conclusive in the kidney. The efficacy of our method with only one cycle of ischemia and reperfusion offers further advantages and brings preconditioning closer to the clinics. In fact, this one-cycle preconditioning schedule has been reported by other groups to be effective in the liver [5, 6] and the small bowel [8], describing protection on the injury associated with ischemia of the organ.

Most of the studies with one-cycle preconditioning schedules only assess ischemic windows while they keep reperfusion unchanged [5, 6]. Our current study also evaluated the reperfusion window, showing that when it was prolonged to 20 or 40 minutes following 15 minutes of ischemia, the preconditioning effect disappeared. It can be argued that this one-cycle schedule is a weak procedure of preconditioning. However, a previous study with a four-cycle schedule, in which the reperfusion time was further increased by 30 minutes, disclosed a similar lack of protection against renal ischemia [12]. These findings conflict with heart studies in which preconditioning induces not only an early protective interval that may extend to one hour, but also a second window of protection at 24 hours after preconditioning [11]. Thus, our results suggest that the interval of protection induced by preconditioning varies according to the organ, and is extremely short in the kidney.

Nitric oxide has been linked to ischemic preconditioning in several organs. To date, in the kidney NO has been associated only with the mechanisms of ischemic damage [22, 26] but its role as innate cellular protector in preconditioning has been scarcely reported. Thus, a rapid generation of renal NO has been evidenced after
Fig. 3. In situ detection of DNA oxidation. Kidneys from warm ischemic study were marked for 8-oxo-2'-deoxyguanosine and examined with fluorescence microscopy. Representative images (x20) are from sham-operated (A), Prec 15/10 (B), and Prec 15/40 (C) groups.

Fig. 5. Effect of preconditioning on cold ischemia. Kidneys from the cold ischemic study were evaluated for conventional histology. Representative images (PAS, x20) are from NoCOLD (A), COLD5H (B), and IP + COLD5H (C) groups are shown.
Table 3. Effects of preconditioning on cold ischemia

<table>
<thead>
<tr>
<th>Score</th>
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<tr>
<td>NoCOLD</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>COLD5H</td>
<td>2.0 ± 0.3*</td>
</tr>
<tr>
<td>IP + COLD5H</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>P</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

Histological evaluation was performed similarly to warm renal studies. Statistical analysis was by Kruskal-Wallis/Connover’s test.

*P < 0.05 COLD5H vs. No COLD and IP + COLD5H

Despite the higher production of renal nitrite and nitrate levels when reperfusion was prolonged to 20 or 40 minutes, the preconditioning effect was not observed. This suggests that the protective window induced by endogenous NO is notably narrow. We thus aimed to elucidate these results. During reperfusion, tissue 8-oxo-2′-deoxyguanosine, a highly sensitive cellular marker of oxidative damage [26], increased with renal nitrite and nitrate levels. Although free radical production is an early and relatively transient event [31], as reperfusion damage advances, tissue structures, including DNA, become progressively oxidized [32]. The higher tissue 8-oxo-2′-deoxyguanosine levels in Prc 15/20 and Prc 15/40 groups reflect the presence of a bulk of free radicals that may oxidize NO and thus inactivate its physiological protective effect. These higher NO levels may thus become cytotoxic, presumably owing to peroxynitrite formation. Furthermore, although a low level of NO may up-regulate anti-apoptosis through protecting molecules such as Bcl-2 and Bcl-xl [33], the interaction between free NO and superoxide initiates an apoptotic process [34] that aggravates ischemic damage. Similar approaches have been reported by studies on hepatic preconditioning [6], but instead of reperfusion, which was kept constant, Peralta et al tested various intervals of warm ischemia. In the liver, the optimal preconditioning window was defined by the balance between the tissue concentration of adenine-NO and xanthine produced during ischemia. Therefore, in our one-cycle preconditioning schedule, the deleterious effect of oxidative damage induced by ischemia prevails over the beneficial effect of endogenous NO release when reperfusion lasts more than 10 minutes.

Pharmacological manipulation with the non-selective NOS blocker also supports the implication of NO in renal ischemic preconditioning. First, renal nitrite and nitrate levels were reduced after L-NAME administration. Second, renal function was not protected, thus indicating that the preconditioning effect had been abolished. And third, the addition of the NO donor to preconditioned animals treated with L-NAME restored the preconditioning effect. As reported elsewhere [26], the use of this non-selective NOS blocker further impaired renal damage and resulted in high mortality, thus confirming that the NO system is a natural defense of cells...
Against ischemic injury. Preconditioning studies in other organs have also shown that NO production is stimulated by endogenous adenosine [14, 20]. During a short ischemia period, there is accumulation of adenosine from adenosine 5'-triphosphate (ATP) degradation and during reperfusion NO is generated as a result of adenosine receptor activation [4]. In the kidney, adenosine and adenosine agonists may contribute to the attenuation of ischemia-reperfusion injury through A<sub>1</sub> or A<sub>2A</sub> receptor activation [14, 35] or A<sub>2A</sub> antagonism [14].

Since the selective blockade of inducible NOS (iNOS) produced not only the early inhibition of nitrite and nitrate generation but also the disappearance of the preconditioning effect, we aimed to determine whether this enzyme is involved in this early phase of ischemic preconditioning. This may be a controversial issue, since the synthesis de novo of iNOS may take longer than the time included in our schedule. Previous experiments have shown that ischemic preconditioning is associated with rapid activation of tyrosine kinases [36, 37] and that iNOS undergoes a post-translational modification via tyrosine phosphorylation, resulting in enzymatic activation as early as 5 minutes after experimental manipulation [38]. Therefore, the iNOS tonically present in the kidney may have been activated by preconditioning and consequently offer renal protection. As the specific blocker was administered early enough to ensure its maximal action, although we did not measure transcription or translation of iNOS, the lowering in renal nitrite and nitrate levels is probably due to a reduction in NO by this pathway. The dose of aminoguanidine used in our study may have inhibited not only iNOS, but also endothelial NOS (eNOS), as the compound is only 10- to 40-fold selective for iNOS versus eNOS [24]. However, to our knowledge all the pharmacological iNOS blockers available are poorly selective or have effects on eNOS. Thus, although our results suggest the participation of iNOS in early renal preconditioning, further experiments, like the use of knockout mice for iNOS or antisense oligodeoxynucleotides, should be performed and carefully discussed.

Ischemic preconditioning was first described as a method to protect the heart from myocardial infarction when sustained ischemia is induced either in minimally invasive percutaneous angioplasty or during anastomosis of the vascular graft [39]. In other organs, preconditioning may be used to study not only innate defenses against ischemic injury but also its great potential for organ preservation [40, 41]. Thus, we evaluated the effect of our one-cycle schedule on cold ischemia in rat renal transplantation, keeping in mind its potential for renal preconditioning in clinical transplantation. Preconditioning improved renal function during the seven-day follow-up and, more importantly, the renal structure was also preserved. Although five hours of preservation is a short cold ischemia time, previous studies showed that it caused severe acute renal failure [27]. In clinical renal harvesting, potential protective maneuvers should be as simple as possible. The one-cycle schedule proposed here thus might be an appropriate procedure in humans. As the ischemia and, especially, reperfusion windows presented are brief enough, ischemic preconditioning may be a suitable procedure in clinical renal transplantation.

In summary, ischemic preconditioning improves the acute renal failure induced by warm and cold ischemia-reperfusion injury. In the rat kidney, 15 minutes of warm ischemia and 10 minutes of reperfusion is the optimal schedule for preconditioning. Our data indicate that the protective effect of renal ischemic preconditioning is related to the local production of NO and that the effect may be lost depending on the balance between NO and oxidative stress induced by preconditioning. This endogenous protective mechanism may be promising in clinical renal preservation.

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REFERENCES

3. Meldrum D, Mitchel MB, Banerjee A, Hariken AH: Cardiac preconditioning can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. J Mol Cell Cardiol 32:1159–1167, 2000


17. MARRER MS, LATCHMAN DS, WALKER JM, YELLON DM: Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 89:1264–1272, 1993


