Renal endothelial dysfunction and impaired autoregulation after ischemia-reperfusion injury result from excess nitric oxide

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Guan, Zhengrong, Glenda Gobé, Desley Willgoss, and Zoltán H. Endre. Renal endothelial dysfunction and impaired autoregulation after ischemia-reperfusion injury result from excess nitric oxide. Am J Physiol Renal Physiol 291: F619–F628, 2006. First published March 28, 2006; doi:10.1152/ajprenal.00302.2005.—Endothelial dysfunction in ischemic acute renal failure (IARF) has been attributed to both direct endothelial injury and to altered endothelial nitric oxide synthase (eNOS) activity, with either maximal upregulation of eNOS or inhibition of eNOS by excess nitric oxide (NO) derived from iNOS. We investigated renal endothelial dysfunction in kidneys from Sprague-Dawley rats by assessing autoregulation and endothelium-dependent vasorelaxation 24 h after unilateral (U) or bilateral (B) renal artery occlusion for 30 (U30, B30) or 60 min (U60, B60) and in sham-operated controls. Although renal failure was induced in all degrees of ischemia, neither endothelial dysfunction nor altered facilitation of autoregulation by 75 pm angiotensin II was detected in U30, U60, or B30 kidneys. Baseline and angiotensin II-facilitated autoregulation were impaired, methacholine EC50 was increased, and endothelium-derived hyperpolarizing factor (EDHF) activity was preserved in B60 kidneys. Increasing angiotensin II concentration restored autoregulation and increased renal vascular resistance (RVR) in B60 kidneys; this facilitated autoregulation, and the increase in RVR was abolished by 100 μM furosemide. Autoregulation was enhanced by Nω-nitro-L-arginine methyl ester. Peri-ischemic inhibition of inducible NOS ameliorated renal failure but did not prevent endothelial dysfunction or impaired autoregulation. There was no significant structural injury to the afferent arterioles with ischemia. These results suggest that tubuloglomerular feedback is preserved in IARF but that excess NO and probably EDHF produce endothelial dysfunction and antagonize autoregulation. The threshold for injury-producing, detectable endothelial dysfunction was higher than for the loss of glomerular filtration rate. Arteriolar endothelial dysfunction after prolonged IARF is predominantly functional rather than structural.

isolated perfused rat kidney; tubuloglomerular feedback; angiotensin II-facilitated autoregulation; endothelium-derived hyperpolarizing factor

There is increasing evidence that endothelial dysfunction in the kidney plays a significant role in ischemia-reperfusion acute renal failure (IARF). Endothelial dysfunction may contribute to the “no-reflow” phenomenon (14, 30, 38) and may explain the observed impairment of autoregulation in IARF (1, 3). Because renal autoregulation controls both renal blood flow (RBF) and glomerular filtration (GFR), loss of autoregulation secondary to endothelial dysfunction in IARF may be central to both reduced GFR and persisting vasoconstriction or enhanced sensitivity of the renal vasculature to vasoconstrictors.

Classically, endothelial dysfunction in the renal and other arterial beds is defined in functional terms as impaired vasorelaxation to endothelium-dependent vasodilators such as acetylcholine (3, 26). Renal endothelial dysfunction has also been defined as impaired nitric oxide (NO) generation in response to bradykinin (33) or acetylcholine (18) or simply as altered availability of NO (43). Morphological and molecular evidence for endothelial injury in IARF has included endothelial cell swelling (10), increased expression of ICAM-1 (20), and Arg-Gly-Asp (RGD)-binding integrins (36). Functional evidence of injury includes videomicroscopic observations of impaired microvascular flow (51) and fluorescence (2-photon) microscopy demonstrations of endothelial cell dehiscence and aggregation, causing renal microvascular obstruction (31, 39). The original suggestion that NO generation by endothelial nitric oxide synthase (eNOS) is impaired in IARF conflicts with evidence for increased eNOS expression and activity (3, 44). However, a reduced response to endothelium-dependent vasodilators has been explained by already maximal activation of eNOS (3), suppression of eNOS-derived NO generation by high-output NO production from inducible NOS (iNOS) (33), and tetrahydrobiopterin (BH4) deficiency with relative NO deficiency (18).

Changes in postglomerular microvascular flow may reflect injury to the microvascular endothelium, but they do not explain loss of autoregulation in IARF, because this is primarily a function of the vasomotor tone of the afferent arteriole (48). Tubuloglomerular feedback (TGF) closely modulates renal autoregulation under normal conditions, but the role of TGF in IARF also remains controversial. Early micropuncture studies by Mason and colleagues (29) suggested that TGF was preserved in IARF, supporting the hypothesis that activation could result in vasoconstriction of the afferent arteriole and decrease GFR with beneficial maintenance of circulating blood volume by prevention of natriuresis despite tubular injury (41). However, TGF inhibition did not correct GFR or reverse oliguria in ischemia-reperfusion (IR) kidneys (19, 28), raising doubt about the functional status of TGF and whether this could be contributing to impaired renal autoregulation or raised renal vascular resistance (RVR) in IARF. Theoretically, increased TGF could contribute to both the enhanced vasoconstrictor hypersensitivity observed with renal nerve stimulation...
and ANG II and the attenuated vasorelaxation in response to acetylcholine and bradykinin (3, 5).

We therefore investigated endothelial function and autoregulation in IARF in the intact kidney using a combined protocol. IARF was induced in rats using unilateral (U) or bilateral (B) renal artery occlusion for 30 (U30, B30) or 60 min (U60, B60), followed by assessment of renal hemodynamics in isolated perfused kidneys 24 h later. Baseline and ANG II-facilitated autoregulation were assessed in response to step increases in perfusion pressure. The integrated response of renal endothelium was assessed in the intact kidney from the vasodilator response to the endothelium-dependent vasodilator methacholine (MCh) after preconstriction by ANG II. We also examined the effect of modulating NO production and inhibiting TGF on autoregulation.

**MATERIALS AND METHODS**

**Materials**

ANG II, phenylephrine, MCh, Nω-nitro-L-arginine methyl ester (L-NAME), l-Nω-(1-iminoethylyl)lysine (l-Nil), furosemide, and indomethacin were purchased from Sigma (St. Louis, MO).

**Induction of IR In Vivo**

All procedures were approved by the Animal Experimentation Ethics Committee of the University of Queensland and were performed in accordance with guidelines of the National Health and Medical Research Council of Australia for animal research. Sprague-Dawley rats weighing from 180 to 220 g were used in all experiments. IR was induced as described previously (12). Briefly, rats were anesthetized with an intraperitoneal injection (60 mg/kg body wt) of pentobarbital sodium (Nembutal, Rhone Merieux). The right renal artery was cannulated with a 22-gauge cannula through the renal artery was cannulated with a 22-gauge cannula through the renal artery. The clamps were occluded with atrumatic microvascular clamps. The clamps were released after 30 or 60 min of occlusion. After 24 h of reperfusion, the right kidney was isolated and perfused in vitro (IPRK) as described below. Sham-operated (control) rats were used to control for the effect of modulating NO production and inhibiting TGF on autoregulation.

**Isolated Kidney Perfusion**

The IPRK preparation was performed as previously described (6). Briefly, rats were anesthetized with pentobarbital sodium. The right renal artery was cannulated with a 22-gauge cannula through the superior mesenteric artery and secured with ligatures during continuous perfusion to prevent ischemia. The kidney was perfused at 37°C with Krebs-Henseleit buffer (KHB) containing 6.7% bovine serum albumin, 5 mM glucose, 20 amino acids, and 1 μM of [14C]inulin and gassed with 95% O2-5% CO2. Renal perfusion pressure was measured directly within the perfusion cannula via a polyethylene line connected to a pressure transducer, and renal pressure during the equilibration period was maintained between 90 and 95 mmHg by a process controller that regulated the speed of the peristaltic pump. Perfusion flow was monitored by wide-beam ultrasound using a Transonic T106 flowmeter (Transonics, Ithaca, NY). After perfusion was initiated, 20 min were allowed for equilibration, after which urine samples were collected at 5-min intervals for assessment of initial kidney function from GFR (urinary clearance of [14C]inulin), perfusate flow, and RVR.

**Experimental Protocols**

**ANG II and phenylephrine dose response.** Dose-response curves for ANG II and phenylephrine were obtained to assess the effect of IR on renal vascular reactivity. Because U60 and B30 kidneys had a similar autoregulatory profile with 75 pM ANG II to kidneys from control and U30 rats, the ANG II dose response was not repeated in the U60 and B30 groups and quantified only in the control, U30, and B60 groups. The phenylephrine dose response was measured in control and B60 group kidneys.

**Autoregulation and endothelial function.** A combined ramp protocol was developed to assess renal autoregulation and endothelial function simultaneously in the IR kidney (Fig. 1). The standard four-ramp protocol used to assess autoregulation (15) was modified to include an MCh dose-response curve as an index of endothelial function (11, 45). As shown in Fig. 1, the perfusion pressure for studying autoregulation was ramped from 70 to 130 mmHg in 15-mmHg steps and then returned to 70 mmHg. Ramp 1 involved no additions and is also referred to as “baseline” or “control” conditions. During ramp 2, 75 pM ANG II was infused into the renal artery. After the maximal step in pressure, the perfusion pressure was continued at 130 mmHg and MCh was infused at concentrations from 10–10 to 10–8 M while ANG II infusion was continued. This allowed determination of the dose response to MCh as an index of the integrated renal endothelial function. Each MCh concentration was infused for 5 min (n = 5–6/group). Note that the process controller was not used to maintain constant pressure during alterations of pressure, or during equilibration after the addition of vasocostrictors such as l-NAME (see below), because this would have exaggerated the reduction in flow under the latter conditions.

**Effect of increased ANG II concentration on autoregulation.** Because infusion of 75 pM ANG II produced only slight enhancement of autoregulation in B60 kidneys (see RESULTS), we assessed whether a higher concentration of ANG II would enhance autoregulation. The standard four-ramp protocol (15) was performed in control and B60 kidneys (n = 5 for each). During ramp 2, a higher concentration of ANG II (200 pM) was infused into B60 kidneys whereas 75 pM ANG II was infused into control kidneys. Infusion of ANG II was then continued during infusion of 50 nM MCh in ramp 3. Ramp 4 was performed after addition of papaverine (0.1 mM).

**TGF.** The presence of TGF was assessed in B60 kidneys (n = 3) by using a maximal inhibitory dose of furosemide. The protocol was the same as the experiment using a 200 pM dose of ANG II during ramp 2, but furosemide (100 μM) was administered instead of MCh before ramp 3 was performed, and then infusion of 200 pM ANG II was continued during ramp 4.

**Effects of endogenous PGs and NO.** The role of endogenous PGs and NO and their effects on ANG II-facilitated autoregulation were examined by nonspecific inhibition of cyclooxygenase (COX) and NOS as previously described (15). Two groups of B60 kidneys (each n = 5) were studied. The first group of kidneys was perfused with 10 μM indomethacin and the second group with both indomethacin and l-NAME (10 μM) 30 min before the standard four-ramp protocol. In all experiments, 75 pM ANG II was infused during ramp 2.

**Endothelial-derived hyperpolarizing factor activity.** The influence of endothelial-derived hyperpolarizing factor (EDHF) activity was studied by infusion of MCh in the presence of combined COX/NOS.
inhibition. As demonstrated in the above experiments, infusion of MCH still antagonized ANG II-facilitated autoregulation in the presence of both COX/NOS inhibition. To ensure complete NOS inhibition, a 10-fold higher concentration of L-NAME (100 μM, n = 4) was used. Ramp 1 was conducted in the presence of 10 μM indomethacin. After ramp 1, L-NAME was added to the reservoir to a final concentration of 100 μM and the kidney perfused for 30 min until pressure and flow were stable. Ramp 2 was then performed. MCH (50 nM) was infused during ramp 3.

Effect of peri-ischemic iNOS inhibition. In five rats, the iNOS inhibitor L-NAME (3 mg/kg) was administered via the femoral vein 15 min before IR followed by infusion (1 mg·kg⁻¹·h⁻¹) throughout the 60-min ischemic period. After 24-h reperfusion, the right kidneys were isolated and perfused for renal autoregulation study using the standard four-ramp protocol with ANG II infused at 75 pM.

Histopathology

The presence of structural damage to the endothelium and vascular smooth muscle cells (VSM) of the afferent arterioles after ischemia was examined using toluidine blue-stained resin sections (1-μm sections). Unperfused right kidneys from control, B30 (n = 4 for each), and B60 (n = 6) rats were flushed with KHB containing 1:1,000 heparin to remove all blood and then perfused with fresh fixative (4% buffered paraformaldehyde) solution for 5 min after cannulation of the renal artery. Fixed kidneys were cut into 1 × 1-mm³ blocks and left in fixative for a further 2–4 h before being washed in PBS and stained. Five sections were examined per kidney by one observer blinded to the groups. Sections were examined at ×40 magnification to count glomeruli and identify arterioles and then at ×400 to assess damage. Where damage was noted, this was confirmed under oil immersion (×1,000). The arteriolar changes of endothelial cell swelling or loss and of VSM swelling were noted; if these features were present, the section was given a score of 1. If absent, a score of 0 was used. In this way, at least 10–30 afferent arterioles/kidney were identified and scored. The proportion of sections with the feature (0/1) for each kidney was used for statistical analysis.

Calculations and Statistical Analysis

Dose-response curves for each vasoactive agent were calculated by nonlinear least squares regression, and the EC₅₀ determined using the PRISM statistical package. Different groups were compared using the residual sum of squares by variance ratio analysis (F-test) (8). Hemodynamic data are presented as means ± SE. A two-way ANOVA was used to identify differences induced by IR of various durations. Differences were analyzed by the Newman-Keuls post hoc test, where P < 0.05 was considered significant. Histological data are presented as median scores and analyzed by the Kruskal-Wallis test.

RESULTS

Renal Function In Vivo and In Vitro After IARF

In vivo SCr concentrations at 0 and 24 h after reperfusion are shown in Table 1. There was no difference in initial SCr among the groups. At 24 h, a significant increase in SCr was seen in the U60, B30, and B60 groups, with the B60 group showing the greatest increase. Initial IPRK functional parameters are also shown in Table 1. In B60 rat kidneys, the perfusate flow was significantly reduced and RVR increased, whereas no significant differences in flow or RVR were seen between any of the other IR and control groups. Initial GFR was significantly decreased in all IR groups at 24 h. The discrepancy between plasma creatinine and unilateral GFR highlights the compensation, which the normal unclamped kidney makes for loss of filtration by the unilateral clamped kidney. In vivo this obscures any rise in plasma creatinine at 24 h with moderate injury (U30 kidneys) but not severe injury (U60), where the loss of total renal function must approach 50%, at least temporarily.

Table 1. Effect of ischemia on serum creatinine and IPRK function

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Creatinine In Vivo, μM</th>
<th>Pressure, mmHg</th>
<th>Flow, ml·min⁻¹·g KW⁻¹</th>
<th>RVR, mmHg·min·g KW⁻¹</th>
<th>GFR, ml·min⁻¹·g KW⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.5 ± 2.5</td>
<td>12.5 ± 2.5</td>
<td>90.5 ± 0.7</td>
<td>30.4 ± 0.9</td>
<td>2.97 ± 0.11</td>
</tr>
<tr>
<td>U30</td>
<td>13.3 ± 3.3</td>
<td>16.7 ± 3.3</td>
<td>90.8 ± 0.7</td>
<td>30.4 ± 1.6</td>
<td>3.05 ± 0.15</td>
</tr>
<tr>
<td>U60</td>
<td>12.5 ± 2.5</td>
<td>27.5 ± 2.9*</td>
<td>94.3 ± 2.1</td>
<td>30.4 ± 0.0</td>
<td>3.18 ± 0.31</td>
</tr>
<tr>
<td>B30</td>
<td>14.0 ± 2.4</td>
<td>28.0 ± 2.0*</td>
<td>90.3 ± 0.6</td>
<td>29.5 ± 1.6</td>
<td>3.10 ± 0.17</td>
</tr>
<tr>
<td>B60</td>
<td>12.5 ± 2.5</td>
<td>148.9 ± 11.1*</td>
<td>92.1 ± 0.6</td>
<td>24.2 ± 0.9†</td>
<td>3.92 ± 0.18†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Percentages of isolated, perfused right kidney (IPRK) function were obtained after equilibration for 20 min after commencement of perfusion U30, U60, B30, and B60: 30- and 60-min unilateral and bilateral renal artery occlusion, respectively; RVR, renal vascular resistance; GFR, glomerular filtration rate; KW, kidney wt. *P < 0.01 vs. 0 h. †P < 0.01 vs. control.
Renal Endothelial Function After IARF

Vasorelaxation in response to the endothelium-dependent vasodilator MCh is shown in Fig. 3 for all groups. Infusion of MCh caused dose-dependent increases in flow in all groups. Vasorelaxation was significantly attenuated in B60 kidneys, as demonstrated by the increase in MCh EC50 (to 40.6 ± 13.6 vs. 17.0 ± 2.9 nM in control kidneys, P < 0.01), demonstrating endothelial dysfunction in this group. There was no difference in EC50 between control and the other IR groups. The maximal vasodilatory response to MCh, expressed as the percent decrease in RVR, was not significantly different among any of the IR or control groups and ranged from 30 to 39%.

Renal Autoregulation After IARF

The pressure-flow relationships for ramps 1 and 2 are shown in Fig. 4. The autoregulatory indexes for all groups are summarized in Table 2. During ramp 1 (no additions, Fig. 4A), the pressure-flow relationship in U30, U60, and B30 kidneys was similar to control and all autoregulatory indexes were <0.7 (Table 2), demonstrating partial autoregulation. B60 kidneys showed impaired autoregulation, with a linear increase in flow over the entire pressure range and an autoregulatory index of 0.95 ± 0.09 (P < 0.01 vs. control). Infusion of 75 pM ANG II (Fig. 4B) in U30, U60, and B30 kidneys caused facilitation of autoregulation between 85 and 130 mmHg, similar to control, with the autoregulatory indexes between 0.03 and 0.20. ANG II (75 pM) also caused significant facilitation of autoregulation in the B60 group (Fig. 4B), decreasing the autoregulatory index from 0.95 ± 0.09 to 0.57 ± 0.10 (Table 3, P < 0.05). Facilitation of autoregulation by 75 pM ANG II in the B60 group remained incomplete compared with control and other IR groups.

Infusion of a higher dose of ANG II (200 pM) facilitated autoregulation in the B60 kidneys (Fig. 5B) that was comparable to sham-operated kidneys (Fig. 5A). The autoregulatory index decreased from 0.97 ± 0.09 under baseline conditions to 0.30 ± 0.07 during ANG II infusion (Table 2, P < 0.01). Infusion of MCh reversed the facilitation of autoregulation by 200 pM ANG II, increasing the autoregulatory index to 1.06 ± 0.13 (P < 0.01 vs. ANG II).

TGF After IARF

The effect of 100 μM furosemide on ANG II-facilitated autoregulation in B60 kidneys is shown in Fig. 6. The enhancement of renal autoregulation by 200 pM ANG II was abolished by 10 μM furosemide. ANG II increased RVR during perfusion at 130 mmHg but not 70 mmHg in B60 kidneys (Table 3). This ANG II-mediated increase in RVR was also abolished by 100 μM furosemide.

Endogenous PGs and Autoregulation After IARF

Addition of 10 μM indomethacin slightly decreased baseline flow rate, but there was no significant difference compared with B60 kidneys without indomethacin (20.8 ± 2.4 vs. 24.7 ± 2.1 ml/min). Neither the pressure-flow relationship (data not shown) nor the autoregulatory indexes (Table 4) were changed by addition of indomethacin to B60 kidneys under baseline condition (no additions, 0.91 ± 0.02 vs. 0.95 ± 0.09) or during ANG II infusion (0.62 ± 0.11 vs. 0.57 ± 0.10).
Modulation of Autoregulation by NO

During equilibration after addition of 10 μM L-NAME for 30 min, flow decreased from 26.4 ± 3.1 to 21.6 ± 3.1 ml/min, whereas perfusion pressure increased from 90.5 ± 1.0 to 164.6 ± 5.3 mmHg. Flow decreased further to 12.8 ± 1.6 ml/min after perfusion pressure was lowered to 70 mmHg. Flow remained almost constant after pressure ramping (Fig. 7A), and the autoregulatory index was 0.28 ± 0.06 (Table 4). L-NAME also enhanced the effect of 75 pM ANG II (Fig. 7B), lowering the autoregulatory index (Table 4) to that observed in control kidneys (0.06 ± 0.07). Enhancement of autoregulation was antagonized by infusion of MCh at 50 nM (Fig. 8), which increased the autoregulatory index to 0.60 ± 0.08 (P < 0.01 vs. ANG II).

EDHF Activity and Autoregulation After IR

The vascular response to infusion of MCh in the presence of combined NOS/COX inhibition is shown in Fig. 9. Giving a

Table 2. Renal autoregulatory index 24 h after ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Ramp 1 (No Additions)</th>
<th>Ramp 2 (ANG II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.65±0.06</td>
<td>0.03±0.07§</td>
</tr>
<tr>
<td>U30</td>
<td>0.59±0.10</td>
<td>0.20±0.08§</td>
</tr>
<tr>
<td>U60</td>
<td>0.54±0.09</td>
<td>0.10±0.10§</td>
</tr>
<tr>
<td>B30</td>
<td>0.61±0.09</td>
<td>0.04±0.09§</td>
</tr>
<tr>
<td>B60</td>
<td>0.95±0.09†</td>
<td>0.57±0.10†‡</td>
</tr>
<tr>
<td>B60 (200 pM ANG II)</td>
<td>0.97±0.09†</td>
<td>0.30±0.07*§</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of kidneys. The autoregulatory index was calculated over the range 85–130 mmHg. ANG II was infused at 75 pM in all experiments except the final B60 group as indicated. *P < 0.05, †P < 0.01 for ischemia-reperfusion (IR) groups vs. control under same conditions. ‡P < 0.05, §P < 0.01 for ANG II vs. no additions in the same group.
higher concentration of l-NAME (100 \( \mu \)M) had no additional effect compared with l-NAME at 10 \( \mu \)M (data not shown), and the autoregulatory index was not changed (0.24 ± 0.03 vs. 0.24 ± 0.06), suggesting complete inhibition of endogenous NOS by 10 \( \mu \)M L-NAME. Enhancement of autoregulation by 100 \( \mu \)M L-NAME in the presence of 10 \( \mu \)M indomethacin was reversed by infusion of MCh (50 nM), demonstrating preservation of EDHF activity in B60 kidneys.

**Inhibition of iNOS After IR**

Peri-ischemic treatment of B60 rats with L-Nil decreased the 24-h SCr from 148.9 ± 11.1 (B60 alone) to 82 ± 11.5 \( \mu \)mol/l (P < 0.05). The pressure-flow relationship under both baseline conditions (no additions) and during ANG II infusion was unchanged by L-Nil treatment. The sensitivity of renal vasculature to MCh was not enhanced by L-Nil treatment; the EC50 of MCh increased to 75.3 ± 29.9 nM; this was not significant vs. ischemia without L-Nil treatment (40.6 ± 13.6 nM, P > 0.05, n = 5).

**Vascular Histopathology**

Even with 60-min bilateral ischemia, afferent arterioles showed little obvious damage by light microscopy compared with control kidneys. Graded assessment of endothelial swelling, cell loss, or VSM swelling revealed no significant differences. Changes in the afferent arterioles of control, B30, and B60 kidneys are summarized in Table 5.

**DISCUSSION**

Recent direct observations have highlighted the role of structural injury to the renal endothelium in IARF (13, 31, 51).
However, the nature and role of endothelial dysfunction in modulating flow through the major renal resistance vessels, the afferent arterioles, are less obvious. A decrease in the transcapillary hydraulic pressure gradient ($\Delta P$) is responsible for $\sim 50\%$ of the characteristic decrease in GFR during delayed graft function following cadaveric renal transplantation, with tubular obstruction and backleak responsible for the remainder (2, 35). The combination of reduced transcapillary hydraulic pressure gradient with impaired renal autoregulation strongly suggests involvement of the renal resistance vessels in the persistent reduction in renal blood flow (23) after IARF. The results reported here highlight the role of endothelial dysfunction in impaired autoregulation after severe IARF but reveal a dissociation between endothelial dysfunction and loss of GFR after moderate IARF.

GFR was reduced significantly 24 h after both unilateral and bilateral renal ischemia. Neither moderate (30 min) nor prolonged (60 min) unilateral ischemia produced detectable ipsilateral endothelial dysfunction or altered facilitation of autoregulation by ANG II. Bilateral ischemia for 30 min also produced no detectable change in these parameters. In contrast, prolonged ischemic insult to the kidney produced by 60-min bilateral artery occlusion resulted in endothelial dysfunction, with a 239% increase in the MCh EC$_{50}$ compared with control ($P < 0.01$). This abolished the partial renal autoregulation seen under baseline conditions and attenuated facilitation of autoregulation by ANG II. The EC$_{50}$ for ANG II, but not phenylephrine, was significantly increased in B60 kidneys and the maximal response to both vasoconstrictors was significantly increased. The maximal vasodilator response to MCh was not significantly different among the groups, and attenuation of ANG II-facilitated autoregulation was improved by increasing the ANG II dose, consistent with the rightward shift in the ANG II dose-response curve. This suggests that the threshold for injury leading to detectable functional endothelial dysfunction is higher than for loss of GFR. The results further suggest that, even after prolonged ischemia, impairment of ANG II-facilitated autoregulation and endothelial function is primarily functional rather than structural in origin.

Either reduction in NOS activity or altered availability of NO, for example, scavenging by increased superoxide, could explain these findings. Conger et al. (3) observed increased expression of cNOS 1 wk after ischemia. Twenty-four hours after ischemia, Kakoki et al. (18) observed increased total eNOS expression, reduced NO production, and reduced expression of dimeric (active) eNOS. The reduction in dimeric eNOS and NO production was reversed by supplementation with tetrahydrobiopterin, an essential cofactor for NO production by both eNOS and iNOS (22, 49). Tetrahydrobiopterin depletion promotes endothelial dysfunction in vessels exposed to ischemia-reperfusion (42), although the kidneys in the perfusion system used by Kakoki et al. (18) would have been quite hypoxic. Their system utilized only crystalloid buffer, and flow was deliberately restricted and held constant at 5 ml/min by preconstriction with phenylephrine to quantify NO production. In our experience, kidneys perfused this way undergo severe hypoxic injury, not only because of the absence of an oxygen carrier such as erythrocytes (9) but also because such kidneys swell rapidly in the absence of an oncotic agent such as albumin, further limiting oxygen delivery (37). Such kidneys would have developed much more extensive necrotic injury than usually seen, even in erythrocyte-free perfusion systems (cf. 9, 24, 25), which may explain the observed tetrahydrobiopterin depletion.

In the experiments reported here, a functionally intact endothelium with respect to both NO and EDHF production is suggested by the persistence of a normal maximal vasodilator response to MCh and by antagonism of ANG II-facilitated autoregulation by MCh equivalent to control under both normal conditions and in the presence of combined NOS and COX inhibition. We have previously demonstrated that ANG II facilitates renal autoregulation by modulating NO in this model, which has high NO under control conditions (15). Nonspecific inhibition of NOS with l-NAME enhanced auto-

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**Table 5. Arteriolar changes after ischemia-reperfusion**

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomeruli Counted</th>
<th>Afferent Arterioles</th>
<th>Endothelial Swelling</th>
<th>Endothelial Loss</th>
<th>VSM Swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control min-max ($n = 4$)</td>
<td>467</td>
<td>162</td>
<td>0.30</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.20–0.40</td>
<td>0.00–0.40</td>
<td>0.00–0.00</td>
</tr>
<tr>
<td>B30 min-max ($n = 4$)</td>
<td>234</td>
<td>70</td>
<td>0.50</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00–1.00</td>
<td>0.00–0.40</td>
<td>0.00–0.60</td>
</tr>
<tr>
<td>B60 min-max ($n = 6$)</td>
<td>301</td>
<td>90</td>
<td>0.63</td>
<td>0.50</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.20–1.00</td>
<td>0.00–0.60</td>
<td>0.00–0.80</td>
</tr>
</tbody>
</table>

Endothelial swelling and loss and vascular smooth muscle cell (VSM) swelling show median score in afferent arterioles demonstrating change. $n$, No. of kidneys examined per group. Five sections were examined per kidney. Arteriolar changes were noted if present and given a score of 1 for that section. If absent, a score of 0 was used. No significant differences were found among the groups.
regulation in B60 kidneys, both under baseline conditions and after infusion of ANG II, demonstrating that excessive rather than deficient NO production contributed to both impaired renal autoregulation and endothelial dysfunction after prolonged ischemia. Preservation of cholinergic sensitivity (via MCh stimulation and antagonism of ANG II-facilitated autoregulation) indicates either that eNOS is not “near maximally upregulated” (3) or that EDHF accounts for a significant part of the response to MCh as occurs in normal kidneys (15). Increased NO production due to activation of iNOS has been shown to contribute to the attenuation of the response to ACh in both afferent and efferent arterioles in LPS-induced ARF (16) and ischemic ARF (33, 34), as well as other pathophysiological changes after IARF (14). Kidneys from iNOS knockout mice are resistant to ischemic injury (27). These studies support a significant cytotoxic role of NO from iNOS in renal ischemic injury but do not necessarily explain the endothelial dysfunction and impaired autoregulation seen in the present studies.

Given that iNOS is upregulated primarily in tubular epithelial cells (34, 52) and that NO produced in this region is likely to enter the venous drainage without passing through the glomerulus, it seems likely that NO-induced impairment of endothelial function and autoregulation after IR is generated from vascular endothelial eNOS and nNOS in either macula densa cells or the renal medulla. This is supported by Thorup and colleagues (40), who suggested that NO generated from renal iNOS did not involve modulation of TGF because inhibition of iNOS with aminoguanidine failed to change glomerular capillary pressure. This is consistent with our observation that peri-ischemic inhibition of iNOS with l-Nil in B60 rats did not ameliorate or prevent impairment of renal autoregulation and endothelial function. Glomerular filtration was partially protected, because plasma creatinine was significantly reduced in this group. With moderate ischemia, there was relative preservation of endothelial function despite a reduced GFR. In severe ischemia, l-NIL reversed this dissociation and protected GFR without preventing endothelial dysfunction. Thus inhibition of eNOS and nNOS by NO derived from iNOS seems unlikely to account for the observed impairment of endothelial function and autoregulation in severe IARF.

Furosemide abolished the enhancement of autoregulation by ANG II and also ANG II-mediated increases in RVR during perfusion at 130 mmHg, suggesting that TGF is intact in B60 kidneys and supporting the suggestion that TGF increases RVR after ischemic ARF. The effect of blocking TGF is likely to be more obvious at higher perfusion pressures in B60 kidneys due to increased GFR and NaCl delivery to the macula densa. At the lowest pressure (70 mmHg), there was no urine output and RVR was higher (Tables 1 and 4), making it unlikely that furosemide could reach the luminal aspect of the macula densa, or have a measurable effect on TGF, because the magnitude of the TGF response is directly proportional to luminal NaCl (e.g., Ref. 21). Thus although we remain uncertain as to how much TGF contributes to increased RVR, our data suggest that it probably does play a role. This is consistent with the micropuncture study by Mason et al. (29), which found a preserved but reduced magnitude of TGF due to the changes in stimulus at macula densa cells in rat kidneys after ischemia. Although hypoxic medullary thick ascending limb injury occurs when this isolated kidney model is perfused without added erythrocytes, we have shown previously that this does not affect our ability to detect TGF (15), presumably because the macula densa is located in the cortex and remains adequately oxygenated. Thus in this model of IARF, the Na-K-2Cl co-transport feedback mechanism for TGF in the macula densa appears intact.

Earlier studies of experimental IARF models showing impairment of autoregulation (1, 50) led to the suggestion that the observed loss of autoregulation and reduction in renovascular reactivity involved necrosis of the VSM cells in the arterial resistance vessels as well as endothelial dysfunction (4). While there was decreased vascular reactivity to ANG II in B60 kidneys evidenced by the increase in ANG II EC50, this appears to have been produced by the excess NO. The maximal vasoconstrictor response to ANG II and phenylephrine was increased, arguing against significant necrosis of VSM cells in the present study. This is supported by the absence of necrosis or severe endothelial or VSM swelling injury on light microscopy. Differences from earlier studies may be accounted for by the prolonged ischemic time (75 min) (4) in contrast to 60 min in the present study.

Although the present study suggests that excess NO production (over the high baseline levels normally present in this model) caused impaired autoregulation after severe ischemia, other pathways could also contribute to this impairment. Compared with kidneys from normal Sprague-Dawley rats (15), the enhancement of autoregulation by l-NAMe appeared incomplete in the B60 group and was not increased by a 10-fold higher concentration of l-NAMe, suggesting that NO was maximally inhibited and that vasodilators other than NO were also actively involved. Possible vasodilators include PGs, EDHF, and peroxynitrite.

Nonselective COX inhibition did not enhance autoregulation in B60 kidneys, suggesting that vasodilator PGs did not contribute significantly to the loss of autoregulation in B60 kidneys or play a role in the early homeostatic response to ARF. Evidence of continued EDHF activity remained after IR injury because MCh reversed ANG II-facilitated autoregulation in the presence of both COX/NOS inhibition, even when the l-NAMe concentration was increased to 10^-4 M. However, as EDHF activity is difficult to quantify, it is unclear whether this represents a normal or altered amount. Oxidative stress is involved in the pathogenesis of renal IR injury (17, 32); superoxide production after IR injury rapidly reacts and inactivates NO to yield other reactive species such as peroxynitrite and hydroxyl radicals, which may account for the cytotoxicity of NO (7, 32, 47). Peroxynitrite can cause vascular relaxation in vivo and alter the VSM response to acetylcholine and prostacyclin (46), and scavenging of peroxynitrite is protective against oxidative injury in IARF (32). Increased EDHF or peroxynitrite production could explain incomplete enhancement of autoregulation by l-NAMe in B60 kidneys.

The evidence for excess NO, preserved EDHF activity, enhanced vasoconstrictor responsiveness, and reversible impairment of ANG II-facilitated autoregulation suggests that these vascular changes in early IARF are functional rather than structural. There is increasing evidence that vascular injury makes a significant contribution to the pathogenesis of the ARF syndrome (13), although the present studies suggest that the threshold for injury leading to endothelial dysfunction is higher than for loss of GFR. The data suggest that eNOS remains
stimulable, although upregulated, at 24 h and that excess NO derived from eNOS and perhaps other endothelium-derived vasodilators such as EDHF contribute to endothelial dysfunction and impaired autoregulation after IARF. Further exploration of the mechanisms by which IR impairs renal autoregulation should be helpful in unraveling the vascular contributions to the maintenance and extension phases of IARF.

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