Loss of the Glomerular Contractile Response to Angiotensin in Rats Following Myohemoglobinuric Acute Renal Failure

BARRY M. WILKES, WILLIAM J.H. CALDICOTT, GERALD SCHULMAN, AND NORMAN K. HOLLENBERG

SUMMARY To examine the state of the glomerulus in rats protected from acute renal failure (ARF) by prior insult, we measured the contractile responses of isolated glomeruli to angiotensin II (AII) and dibutyryl cyclic AMP (DBcAMP). In healthy rats, both agents induced a dose-related fall in glomerular diameter ($P < 0.001$). Saralasin, the angiotensin antagonist, blocked the glomerular response to AII totally and to DBcAMP partially. Two weeks following ARF induced with 50% glycerol (10 ml/kg, im), azotemia had reversed and the nephrotoxic effect of mercuric chloride (4.7 mg/kg, sc) was blunted, as anticipated. ATT did not reduce glomerular size, but the response to DBcAMP was sustained at this time. To determine the specificity of the loss of the glomerular response to AII, we also assessed the effects of an AII infusion (1 μg/kg/min) on blood pressure and renal blood flow. There was a substantial rise in blood pressure (control, 104 ± 11.8 mm Hg; AII, 131.0 ± 5.3 mm Hg; $P < 0.001$) and fall in renal blood flow (control, 2.45 ml/g per min; AII, 0.81 ± 0.10 ml/g per min; $P < 0.025$). Vascular responsiveness to AII was preserved at a time when glomeruli were totally unresponsive and rats were resistant to ARF. The loss of glomerular contractility may, in part, account for the protection from ARF seen in this model: if so, glomerular abnormalities may play a pathogenetic role.


RECENT studies have pointed to intraglomerular events as a major determinant of glomerular filtration (Baylis and Brenner, 1978) and, at least in some models, of the pathogenesis of acute renal failure (Coelho et al., 1973; Daugharty et al., 1974; Cox et al., 1974; Blantza, 1975). For that reason we employed a variant of the technique devised by Sraer et al. (1974) for assessing glomerular contractility in response to known agonists in the model of acute renal failure which follows glycerol. To our surprise, 2 weeks after insult, when azotemia disappeared, glomerular responses to angiotensin could not be elicited. Because one reproducible method for protecting animals against acute renal failure, in at least some models, involves prior insult and protection lasts for at least 2 weeks (Hayes et al., 1970; Oken et al., 1975), we undertook to examine the factors responsible for the loss of glomerular responsiveness to angiotensin.

Methods

In Vivo

Studies were performed in 154 male Sprague-Dawley rats weighing 168-305 g. Standard Purina Rat Chow, with a 0.42% sodium and 1.08% potassium ash content, was provided until just prior to the experiments. Free access to water was allowed except where noted otherwise. Anesthesia was induced with pentobarbitol sodium (40 mg/kg, ip) and maintained with additional doses as required to sustain light anesthesia. An airway was provided by tracheostomy. In studies in which blood flow was measured with microspheres, left ventricular catheterization was achieved by way of the right carotid artery with a lubricated polyethylene (Clay Adams PE 50) catheter. Additional catheters of the same size were placed into the femoral artery for blood pressure monitoring and arterial sample collections, and into the jugular vein for the infusion of drugs. Left ventricular and arterial blood pressures were monitored from the two catheters with Statham P23 Dc transducers and recorded continuously on a Grass model 5 polygraph.

Renal blood flow measurements were made with a radioactive microsphere method as described in detail for this laboratory (Bartrum et al., 1974). In brief, about 100,000 microspheres (15 μm in diameter: New England Nuclear), suspended in a sucrose solution having a specific gravity of 1.3 were infused into the left ventricle in a 0.25-ml volume over 15 seconds and followed by a 0.25-ml saline flush. Approximately 1 ml of arterial blood was collected during the following minute from the femoral artery catheter by free flow to provide the “reference sample” for blood flow calculation. The rats then were killed by air embolism and both kidneys were removed, decapsulated, weighed, and placed in for-
malin for counting emission in a Nuclear-Chicago γ well counter.

Acute renal failure (ARF) was induced with glycerol (50% vol/vol, 10 ml/kg, im) in rats deprived of water for 24 hours. At the appropriate times, blood urea nitrogen concentration (BUN) was measured by the Autoanalyzer method on 0.3-ml blood samples collected from the rat tail vein. To demonstrate protection from ARF afforded by prior ARF, 18 rats were injected with mercuric chloride (4.7 mg/kg, sc) 2 weeks after the glycerol injection. An additional group of 12 age and weight-matched controls that had not received glycerol was also injected with mercuric chloride. When normal rats were given mercuric chloride, BUN increased to 164 ± 12 mg/ml at 24 hours and to 318 ± 28 mg/100 ml at 48 hours. In rats with myohemoglobinuric acute renal failure 2 weeks before mercuric chloride injection, BUN rose from 32 ± 4 mg/100 ml to 141 ± 17 mg/100 ml and to 135 ± 34 mg/100 ml at 24 and 48 hours, respectively. All the rats without prior glycerol-induced ARF had died by the 5th day; 67% of the rats with prior ARF were alive on day 5 (P < 0.001). These results confirm relative protection against ARF by prior ARF (Hayes et al., 1970; Oken et al., 1975).

In Vitro

The procedure for assessing the glomerular response to angiotensin in the rabbit developed by Sraer et al. (1974) was modified in this laboratory, as described in detail (Caldicott et al. 1981). In brief, anesthesia was induced with ether. Bilateral nephrectomy was performed without renal artery perfusion immediately after anesthesia was induced. The kidneys from two rats were used for each assay. The outer cortex was dissected with a surgical scalpel, cooled rapidly at 0°C on ice, and minced with a razor blade. The resultant paste then was processed with a phosphor bronze sieve (#170, with opening 0.0035 inches, W.S. Tyler, Inc.). The suspension was centrifuged at 1200g for 90 seconds, the supernatant discarded, and the pellet resuspended six times. The final pellet containing isolated glomeruli, freed of Bowman's capsule and arterioles, was suspended in 40 ml of the same medium, and 0.9-ml aliquots were measured into separate test tubes. About 60 minutes was required from induction of anesthesia to final separation of the glomeruli.

Glomeruli from three pairs of rats, 24 hours after administration of glycerol, were prepared as described above. In two of the three assays, only glomerular debris could be isolated; in the third only a small number of markedly deformed glomeruli could be identified. At 24 hours after glycerol, glomeruli appear to be severely injured, making them too fragile for in vitro isolation by this technique.

Angiotensin II (Bachem Inc.), the octapeptide analog, saralasin [Sar¹, Ala⁸]-angiotensin II, (Norwich Pharmaceuticals) and dibutyryl cyclic adenosine monophosphate (DBcAMP) were added to the glomerular suspension to produce a final concentration range as indicated in Tables 1 and 2. The optimal concentration of these agents (10⁻¹⁰ M AII and 10⁻⁷ M DBcAMP) was selected for additional experiments (Table 2) on the basis of the dose-response curves. Samples were incubated for 15–20 minutes at room temperature and then subjected to fixation with 1% glutaraldehyde.

Measurements of glomerular diameter were made on a coded basis to avoid potential bias. The diameters of the isolated glomeruli were measured under a light microscope at 100× power (Nikon), with a television camera and video display in place of the eyepiece to facilitate measurement. The system was equipped with a metrology unit which made the measurements in the focal plane of the microscope. The results are expressed as changes in glomerular size (ΔR) with dibutyryl cyclic AMP concentration (Table 2).

**Table 1** Relation of Dose to Glomerular Response to Angiotensin and Dibutryl Cyclic AMP Concentration

<table>
<thead>
<tr>
<th></th>
<th>Change in glomerular size (ΔR) with angiotensin II</th>
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<tbody>
<tr>
<td>10⁻¹⁰ M</td>
<td>10⁻⁹ M</td>
</tr>
<tr>
<td>Normal</td>
<td>-10.6 ± 2.3</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
</tr>
<tr>
<td>2 weeks after ARF</td>
<td>-0.2 ± 2.1</td>
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<tr>
<td>n</td>
<td>5</td>
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<tr>
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<tr>
<td>10⁻¹⁰ M</td>
<td>10⁻⁹ M</td>
</tr>
<tr>
<td>Normal</td>
<td>-3.1 ± 0.4</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
</tr>
<tr>
<td>2 weeks after ARF</td>
<td>2.7 ± 1.5</td>
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<tr>
<td>n</td>
<td>5</td>
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</table>

n represents the no. of rats studied.
TABLE 2  Influence of Saralasin on Glomerular Responses

<table>
<thead>
<tr>
<th></th>
<th>No drug</th>
<th>Sar</th>
<th>All</th>
<th>All + Sar</th>
<th>DBcAMP</th>
<th>DBcAMP + Sar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal n</td>
<td>156.5 ± 1.0</td>
<td>148.0 ± 1.3*</td>
<td>112.8 ± 11</td>
<td>148.1 ± 0.8*</td>
<td>119.4 ± 1.0</td>
<td>129.2 ± 1.2*</td>
</tr>
<tr>
<td>2 weeks after ARF n</td>
<td>144.8 ± 1.5</td>
<td>146.5 ± 1.8</td>
<td>142.2 ± 15</td>
<td>151.6 ± 1.7*</td>
<td>131.8 ± 1.6</td>
<td>133.0 ± 1.5</td>
</tr>
</tbody>
</table>

Values are the mean glomerular diameter ± SEM in microns. Abbreviations are Sar, saralasin; All, angiotensin II; DBcAMP, dibutyryl cyclic adenosine monophosphate. n — the number of rats studied.

* P < 0.001 when compared to the same group without SAR.

system (Circon Electronics). Because glomeruli were often not circular, measurement was made in widest diameter in the horizontal plane of the microscope’s stage, which was an arbitrary function of the position of the slide. Despite this convention, the coefficient of variation in glomerular diameter measurement was always less than 10%. At least 33 glomeruli were measured in each assay tube.

Protocols

Peripheral and Renal Vascular Reactivity to Angiotensin II

The peripheral vascular and renal vascular responsiveness to angiotensin II (Hypertensin; Ciba) was determined in nine normal rats and six rats studied 2 weeks after myohemoglobinuric acute renal failure. Renal blood flow was determined following a 15-minute intravenous infusion of angiotensin II (1 ng/kg per min). Mean blood pressure was recorded throughout the infusion and served as an index of the total vascular response. A control group of 14 rats received an infusion of vehicle alone (normal saline).

Glomerular Responsivity

The in vitro response of isolated glomeruli to the addition of angiotensin II, saralasin, DBcAMP, angiotensin II plus saralasin, and DBcAMP plus saralasin to the incubation medium was determined in 15 normal rats and in 11 rats studied 2 weeks after myohemoglobinuric acute renal failure. As a control for the assay procedure, normal rat glomeruli prepared at the same time as the glomeruli from the experimental groups were studied with each assay.

Statistics

Mean values are presented with the standard error of the mean as the index of dispersion. Student’s t-test was used to assess the statistical significance of differences in renal blood flow, blood pressure and glomerular responsivity. Where appropriate, χ² with a correction for continuity, and the Fisher exact test were used. The null hypothesis was rejected when P was less than 0.05.

Results

Glomerular size showed a Gaussian distribution in all groups (Fig. 1). Addition of angiotensin II to glomeruli derived from healthy rats resulted in a substantial, dose-related (Table 1) reduction in mean glomerular diameter, with the maximum response occurring to the 10⁻¹⁰ M dose. Normal glomeruli also showed a striking response to DBcAMP (Tables 1 and 2; Fig. 2): Saralasin employed alone also induced a small reduction in glomerular size (P < 0.001; Table 2). When superimposed on angiotensin II, saralasin totally blocked the glomerular response to angiotensin II. Saralasin also had a small influence on the glomerular response to DBcAMP; DBcAMP reduced the size of healthy glomeruli in the same experiments to 119.4 ± 1.0 μm. The addition of saralasin significantly limited the reduction in glomerular size induced by 10⁻⁷ M DBcAMP, to 129.2 ± 1.2 μm (P < 0.001).

Two weeks after glycerol-induced acute renal failure, angiotensin was ineffective in reducing mean glomerular diameter (Table 1; Fig. 1); treatment with saralasin did not modify glomerular size (144.8 ± 1.5 vs. 146.5 ± 1.8 μm). When saralasin was superimposed on angiotensin II, glomerular size was 148.1 ± 0.8 μm—not different from saralasin alone. The response to DBcAMP was still present, but reduced; glomerular size fell from 144.8 ± 1.5 to 131.8 ± 1.6 μm; P < 0.001 (Table 1; Fig. 2). The in vivo experiments on pressor and renal vascular responses to angiotensin II were undertaken to assess the specificity of the loss of glomerular responses to angiotensin II (10⁻¹⁰ M). The abscissa represents glomerular diameter in microns (μm). Normal glomeruli show a marked reduction in size in response to angiotensin II, P < 0.001 (left). Two weeks after glycerol-induced acute renal failure, there is no response to angiotensin II (right).
renal failure clearly does not reside in a nonspecific loss of vascular responsiveness to potential mediators: the renal vascular response to angiotensin II, for example, was still striking despite the reduced baseline flow.

The role of glomerular injury in the pathogenesis of acute renal failure remains controversial: glomeruli are generally normal by light and electron microscopy, although scanning electron microscopy has revealed abnormalities in some (Cox et al., 1975) but not all circumstances (Langlinais and Merrill, 1977). Moreover, physiological studies on the glomeruli available for puncture in the Munich-Wistar rat have revealed abnormalities (Daugharty et al., 1974; Blantz, 1975). Further support for glomerular injury in the early phase of acute renal failure was demonstrated: our attempts to isolate glomeruli by sieving techniques often resulted in their total disruption.

By 2 weeks, however, the glomeruli were morphologically intact. Glomerular filtration rate was not assessed directly but was sufficient to maintain a normal blood urea nitrogen. Furthermore, there was no apparent disruption of glomeruli during the sieving techniques. Challenge with pharmacological agents at this time, however, revealed a profound abnormality. Glomeruli were completely unresponsive to angiotensin II but showed a significant, although reduced, response to DBcAMP. Loss of responsiveness to angiotensin II was specific for the glomerulus as the pressor and renal vascular responses were maintained.

Dibutyryl cyclic AMP was employed as a second approach to the specificity of the loss of the glomerular angiotensin II receptor. This agent is likely to act at an intracellular locus, whereas angiotensin II receptors generally are located in the membrane of the effector cell (Gilman and Murad, 1975). Both agents have been shown by micropuncture of superficial glomeruli to exert an intraglomerular action which influences filtration (Blantz et al., 1976; Ichikawa and Brenner, 1977).

Changes in glomerular size probably reflect mesangial contraction. Bernick (1969) demonstrated contractile activity in elements of glomeruli in tissue culture. A highly specific antiserum to human smooth muscle actomyosin has been shown to have great affinity for the mesangial contractile elements, providing an immunochemical link between the contractile elements of smooth muscle and the mesangium (Becker, 1971). Becker suggested that mesangial contraction may play an important role in regulating glomerular blood flow (1971). Hornycz et al. (1972) attributed a dose-related constriction of glomerular capillaries to angiotensin-mediated activation of the mesangial contractile elements, an observation recently confirmed and extended (Au-}

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**Figure 2** *Mean (± SEM) responses of glomeruli to angiotensin II (10^{-10} M) and dibutyryl cyclic AMP (10^{-7} M). Note the loss of response to angiotensin and the attenuated, but still significant, response to dibutyryl cyclic AMP, 2 weeks after glycerol-induced acute renal failure.*

**Figure 3** *Effect of angiotensin II (1 μg/kg per min) on mean blood pressure and renal blood flow in normal rats and in rats 2 weeks after glycerol-induced acute renal failure.*
meruli in vitro (Sraer et al., 1974) and for the fall in the ultrafiltration coefficient (Blantz et al., 1975), \( k_f \) which can be reversed by saralasin (Steiner and Blantz, 1979). Similarly, DBcAMP has been shown to lower \( k_f \) (Ichikawa and Brenner, 1977).

A less likely alternative is that the fall in glomerular size with angiotensin is due to a change in membrane permeability. However, the experimental conditions did not provide an obvious osmotic stimulus to water flux.

What might be the physiological consequences of angiotensin-induced reduction in glomerular size? The net transglomerular passage of substances is proportional to the ultrafiltration coefficient, \( K_f \), which is the product of the intrinsic filtering characteristics of the glomerular capillary and its surface area (Baylis and Brenner, 1978). It seems likely that the very large change in glomerular diameter we documented in response to angiotensin in the normal animal would be reflected in profound effects on glomerular capillary surface area. In ARF, the ultrastructure of the glomerular capillary wall has been altered only minimally in those studies in which any abnormalities could be found (Cox et al., 1975; Langlinais and Merrill, 1977), but \( K_f \) has been reported to be markedly reduced in some models (Blantz, 1975). The major change causing the low \( K_f \) could well be a reduced glomerular capillary surface area (Avasthi et al., 1980).

Current theories of tubulo-glomerular feedback suggest local control mechanisms, which could easily involve the glomerulus (Mason, 1976). When the effects of a spontaneous increase in filtration rate are mimicked by perfusing the loop of Henle at rates higher than normal, single nephron glomerular filtration rate decreases (Schnermann et al., 1972). The local production of angiotensin by the juxtaglomerular cells is believed to mediate the fall in GFR. In acute renal failure, tubuloglomerular feedback may be operative, the net effect of which is to prevent excessive fluid losses from damaged nephrons (Mason, 1976). The common assumption has been that the feedback loop operates by regulating the afferent arteriole; the possibility that tubuloglomerular feedback involves an action directly on the glomerulus must also be considered.

Maximal recovery from ARF takes several weeks, a time during which glomerular responsiveness to angiotensin II and DBcAMP appears to be altered. A reasonable speculation is that these altered glomerular responses may be responsible, at least in part, for protection from ARF.

A possible role for the renin-angiotensin system in the pathogenesis of ARF remains unsettled. On the one hand, plasma renin levels are often elevated in ARF (Brown et al., 1970; Flammenbaum et al., 1972; Tu, 1974). Chronic salt loading which suppresses the renin-angiotensin system protects against ARF (Dibona et al., 1971) and, at least in one study, converting enzyme inhibition prevented ARF (Lindner and Cutler, 1971). On the other hand, a variety of manipulations, all of which block the effects of circulating renin, do not attenuate the severity of experimental ARF (Oken et al., 1975; Ishikawa and Hollenberg, 1976). Bidani et al. (1979) recently demonstrated that sodium chloride-induced protection against nephrotoxic ARF was completely independent of circulating or renal levels of renin, but they did not look at the early initiation phase of ARF.

There are several mechanisms which could possibly account for the loss of the glomerular contractile response to angiotensin. The first was that the contractile process or excitation-contraction coupling had been disrupted. To assess that possibility, we employed a second agent, dibutyl cyclic AMP, which has been shown to influence glomeruli directly (Ichikawa and Brenner, 1977) and which is likely to have an intracellular locus of action. To the extent that the response to DBcAMP was reduced 2 weeks after administration of glycerol, an abnormality in the contractile apparatus or in the coupling mechanism could have accounted for part of the loss of response to angiotensin, but clearly cannot account for the entire disappearance of the response. As a second possibility, a more specific loss of response to angiotensin is possible. This could be due to disruption of a membrane in which the angiotensin receptors reside, a more specific loss of the receptor, a discrete abnormality of excitation-contraction coupling, or occupation of receptors by large amounts of endogenous angiotensin. The last possibility seems unlikely in view of the limited response of the isolated glomeruli to saralasin in this study. Nothing in the data presently available would allow one to choose between the alternatives, but at least several are amenable to direct testing. For example, tracer kinetic studies with radioactive angiotensin would make it possible to assess specific binding to the angiotensin receptor.

The loss of the glomerular response to angiotensin during the interval when protection occurs could well account for the protection. If failure of the glomerulus to contract indeed accounts for the protection, it is difficult to escape the conclusion that the glomerular response participates in the pathogenesis through an influence on surface area or pore size (Avasthi et al., 1980), at least in this model. The data are also compatible with a normal local role for angiotensin, but that, too, remains unproved.

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