Effects of enhanced oxygen release from hemoglobin by RSR13 in an acute renal failure model

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Background. Acute renal failure is believed to be caused, in some circumstances, by impaired oxygen delivery to the outer medulla. This study examined the effect of RSR13, a synthetic allosteric modifier of hemoglobin oxygen-binding affinity, on renal function in a setting of acute renal failure in rats.

Methods. An in vivo model of acute renal failure in the rat produced by reduced renal mass, salt restriction, volume depletion, prostaglandin inhibition, and radiocontrast administration was used. Simulations explained this finding under conditions of severe medullary hypoxia. Mechanistic studies demonstrated marked worsening of medullary hypoxia following RSR13 under conditions similar to our experimental model. Furosemide pretreatment to reduce the imbalance between oxygen supply and demand markedly attenuated the basal-medullary hypoxia produced in the presence of indomethacin and RSR13 (P < 0.01). Additionally, 31P NMR studies demonstrated renal adenosine 5'-triphosphate (ATP) depletion in rats with acute renal failure treated with RSR13 (45% decrease, P < 0.01); again, this effect of RSR13 was completely prevented by pretreatment with furosemide.

Conclusions. Under conditions of severe renal medullary hypoxia, induced in part by indomethacin-mediated reductions in outer medullary blood flow, the administration of RSR13 can exacerbate acute renal dysfunction. However, reducing the rate of oxygen consumption by inhibiting sodium transport with furosemide pretreatment or post-treatment appears to be functionally protective.

Key words: RSR13, allosteric modifier of hemoglobin, oxygen, medulla, cortex, kidney, ischemia, hypoxia.
Acute renal failure studies

Group 1: Effect of RSR13 on acute renal failure. To assure that the volume depletion was achieved, furosemide was administered at a dose of 2 mg/kg (intraperitoneally, IP) for four days followed by a seven-day period in which the rats were permitted to eat only boiled rice as a food source. The combined effect of furosemide and the low-sodium rice diet ensured sodium and volume depletion [6]. All rats had free access to water throughout the study. On the last day of rice consumption, each rat was placed in a metabolic cage to collect a 24-hour urine sample. Subsequently, urine concentrations of creatinine, urea nitrogen, sodium, and potassium were measured; these data together with a plasma sample (discussed later in this article) permitted calculation of baseline creatinine clearance and electrolyte excretion in each rat.

Each rat was weighed and given an intraperitoneal injection of indomethacin (10 mg/kg). Forty-five minutes later, when kidney blood flow is known to be reduced by indomethacin treatment, either RSR13 (150 mg/kg, at 30 mg/mL in normal saline, N = 6) or an equal volume of 0.6% saline was administered via the femoral vein catheter. After one hour, the femoral vein catheter was removed. The vein was ligated, and the incision was closed as in group 1. The rats were returned to the metabolic cages to enable 24-hour urine collections per day over the next three days.

Mechanistic studies

These studies were performed to test directly the hypothesis that there were changes on PO2 in the outer medulla under condition of our experiment and that such changes would correlate with altered production of adenosine triphosphate (ATP), an important component of the oxygen supply-and-demand theory. High-energy phosphates were measured using 31P (phosphorous 31) NMR. These NMR studies were performed using a 7.05 Tesla vertical bore cryomagnet with AM300 spectrometer (Bruker Instruments, Billerica, MA, USA). Detection of the 31P signal was accomplished with a custom-fabricated, two-turn surface coil of 8 mm diameter placed alongside the greater curvature of the kidney after exposure with a flank incision. A jugular vein was cannulated for drug administration during these studies. The probe was tuned to the frequency of 31P (121.5 MHz), and the animal was placed in a specially constructed plastic holder. The rat and holder were placed in a vertical position within the magnet. Following shimming on the proton signal, 31P NMR spectra were acquired using a tip angle of 15 degrees (at the center of the coil), a relaxation delay of 0.051 seconds, and an array size of 1 K points. Five minutes of acquisitions were averaged prior to exponential multiplication with 30 Hz line-broadening, Fourier transformation and phase correction. These spectral data were then analyzed with an automated line-fitting program written in our laboratory [8]. An external standard consisting of methylene diphosphonate in a capillary tube was used to correct for any changes in coil sensitivity.
that might occur during experiments. Tissue ATP content is presented as values relative to the value obtained prior to any experimental manipulations. Intracellular pH values calculations based on the chemical shift of inorganic phosphate were performed as reported previously [9].

**Model of oxygen tensions along the nephron**

Our previous study reported a model for renal oxygen tensions that allows for computer simulation of the effects of altered renal blood flow, transport “work,” and gas exchange between the descending and ascending vasa rectae on renal oxygen and carbon dioxide tensions [10]. For the purpose of this current study, we altered the original program to allow for manipulation of the hemoglobin-oxygen-binding affinity curve. In the simulation program, this sigmoidal curve was generated by the equation 

$$y = \frac{z^2}{(z^2 + k^2)},$$

where $y$ is the oxygen saturation ($O_2$ sat), $z$ is the partial pressure of oxygen (pO$_2$), and $k$ is that pO$_2$ at which the hemoglobin is 50% saturated. This $k$ is referred to in our article as the p50. The modifications to the previously published routine that runs in the software package program Matlab™ (The MathWorks Inc., Natick, MA, USA) were, therefore, limited to variations in the value of the p50.

**Renal pO$_2$ measurements**

Renal pO$_2$ was measured using a needle electrode (No. 758-25; Diamond General Corp., Ann Arbor, MI, USA) coupled to a microprocessor, as previously described [4]. All data were visualized on the LED display of the microprocessor and recorded on a PC for future analysis by an investigator blinded to the treatment. Rats were anesthetized with sodium pentobarbital (30 mg/kg). A jugular vein catheter was inserted for delivery of infusates or additional anesthesia, and the left kidney was exposed and exteriorized through a flank incision. The bladder was catheterized to collect urine.

Partial pressure of oxygen (pO$_2$) was measured under control conditions with the oxygen electrode in the cortex for two minutes at a depth of no more than 1 mm. Then the electrode was advanced deeper into the kidney parenchyma until an abrupt decrease in pO$_2$ was noted; usually this occurred at 3 mm or deeper (depending on the size of the kidney). This is an approximation of the transition zone from cortex to medulla. The electrode was then withdrawn approximately 50 μ, and this location was defined as the outer medulla (OM) [2]. After three to five minutes of steady-state recording of pO$_2$ in the OM, one or more pharmacological agents were given as follows.

Indomethacin (10 mg/mL in 100 mmol/L phosphate, pH 8.0; 10 mg/kg) was added to the abdominal cavity. Twenty minutes later, furosemide (N = 8) was injected via the jugular vein catheter at a dose of 8 mg/kg. Twenty minutes later, RSR13 (N = 8) also was administered via the jugular vein at a concentration of 30 mg/mL of 0.6% saline solution for a dose of 150 mg/kg over several minutes. In the furosemide experiments, 1.0 mL of warm 0.6% saline was given intravenously at 15 minutes to replace urine losses. OM pO$_2$ values are reported as mean ± SEM at 20 minutes after the completion of treatment with indomethacin, furosemide, saline, or RSR13. After these values were obtained, mannitol was given slowly as 2.0 mL of a 5% solution in distilled water, which was approximately a 300 mOsm solution. OM pO$_2$ was again recorded at 20 minutes after completing the mannitol infusion.

**Renal $^{31}$P NMR studies**

Thirty minutes prior to placing the animal in the magnet, indomethacin (10 mg/mL in 100 mmol/L phosphate, pH 8.0, 10 mg/kg) was added to the abdominal cavity through an intraperitoneal catheter, as described previously in this article. In some experiments, furosemide (N = 8) was injected via the jugular vein catheter at a dose of 8 mg/kg at the same time as the indomethacin was administered intraperitoneally. RSR13 (N = 8) was given intravenously as 30 mg/mL of 0.6% saline solution, 150 mg/kg during acquisition of spectral data through the jugular venous catheter over several minutes.

**Statistical analysis**

Data were compared using one- or two-way analysis of variance (ANOVA) and the unpaired or paired Student $t$ test with Scheffe’s correction for multiple comparisons, depending on the unpaired or paired nature of the data [11]. Statistical analysis was performed using SIGMASTAT™ Software (Jandel Scientific, Corte Madera, CA, USA).

**RESULTS**

**Effects of kidney mass reduction and volume depletion**

All rats exhibited rather high hematocrit, as expected from the combined furosemide and salt-restricted rice diet regimen, and the daily urine sodium and potassium excretion rates were low. However, creatinine clearance and serum creatinine were normal, and there were no differences in urine flow rate. Blood urea nitrogen (BUN) values were clearly below those normally observed in rats consuming conventional rat chow that was high in protein (Table 1).

**Effect of RSR13 or vehicle after radiocontrast**

In vehicle-treated rats, there were no changes in creatinine clearance, serum creatinine, or BUN from pretreatment values over the three days following administration of radiocontrast. This is likely due to the rather large
volume of saline given to match the volume of RSR13 solution. However, each rat given RSR13 exhibited a transient elevation of serum creatinine and BUN and a decrease in creatinine clearance. By the third day, renal function returned to pretreatment values in the RSR13-treated group. Urine sodium excretion increased on the first day post-radiocontrast administration in both groups of rats, but the increase was more pronounced in the RSR13-treated animals. By the third day, urine sodium was greater in the vehicle-treated rats (Table 1).

**Effect of furosemide on acute renal failure**

As noted in Table 1, RSR13 caused a transient increase in serum creatinine and BUN and a significant decrease in creatinine clearance at day 1 in this model (all P < 0.05; serum creatinine and creatinine clearance data shown in Fig. 1). In the RSR13 plus furosemide-treated group, the serum creatinine did not increase nor did creatinine clearance decrease significantly at any time during the three days of observation (Fig. 1). However, when the rats were exposed to RSR13 in the absence of indomethacin, RSR13 treatment was not associated with an increase in serum creatinine or decrease in creatinine clearance at any time (Fig. 1).

**Model of oxygen tensions along the nephron**

For the purposes of this simulation, blood flow was allowed to vary; oxygen consumption was held constant at 8 μmol/min, and the equilibrium constant for oxygen exchange was set at 1 × 10⁻⁷ mmol/min/torr as reported previously [10]. Our current report presents vasa rectae blood flow rates ranging from 0.07 to 1.4, which probably covers the physiological range, but we ignored the complex and considerable dependence that metabolic rate has on the glomerular filtration rate that, in turn, is dependent on blood flow (Figs. 2 and 3). When the p50 for hemoglobin is increased from 38 to 48 mm Hg (values reported in the absence and presence of RSR13) and vasa rectae blood flow is high, our model predicts very high oxygen tensions in the medulla. This, in fact, is what prompted us to undertake the study of a possible increase in serum creatinine and BUN and a decrease in creatinine clearance at any time (Fig. 1).

<table>
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<tr>
<th>Table 1. Plasma and urinary characteristics prior to and following RSR13 (N = 6) or vehicle control (N = 4)</th>
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<tr>
<td><strong>Serum creatinine mg/dL</strong></td>
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<td><strong>BUN mg/dL</strong></td>
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<td><strong>Creatinine clearance mL/min</strong></td>
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<td><strong>Na⁺ excretion μEq/day</strong></td>
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<td><strong>Urine flow μL/min</strong></td>
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<td><strong>Serum Na⁺ mEq/L</strong></td>
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<td><strong>Serum K⁺ mEq/L</strong></td>
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<td><strong>Hematocrit vol %</strong></td>
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<td>RSR13</td>
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*Values expressed as mean ± SEM.
един P < 0.05
един P < 0.01 vs baseline
един P < 0.05 vs. control
Reducing the metabolic activity of the thick ascending limb transport segments of the nephron of the kidney by 80% (to simulate an effect of furosemide) raises oxygen tensions dramatically throughout the nephron over the range of blood flows examined (Fig. 3).

**Electrode pO₂ measurements**

The direct measurements of oxygen tension were used to confirm the predictions made by the modeling of this extremely complex vascular bed within the kidney. The data show that in the anesthetized rat, oxygen tension in the cortex was twofold higher than that in the outer medulla (Table 2). pO₂ averaged 24.0 ± 1.2 mm Hg in the cortex and was quite similar among groups (Table 2). Indomethacin treatment decreased medullary pO₂ from 11.2 ± 1.4 to 2.5 ± 0.6 mm Hg (P < 0.01; Table 2). The effect of a subsequent injection of mannitol in these animals was not examined. Furosemide resulted in an increase in medullary pO₂ from 14.6 ± 2.5 to 37.0 ± 6.1 mm Hg (P < 0.01). The subsequent addition of mannitol did not significantly change medullary pO₂, averaging 38.1 ± 6.0 mm Hg.

RSR13 alone did not change the outer medullary pO₂. Medullary pO₂ averaged 10.1 ± 2.0 mm Hg before RSR13 and 11.8 ± 4.1 mm Hg at 20 minutes after RSR13. After mannitol injection, in these RSR13-treated rats, medullary pO₂ remained constant, averaging 12.3 ± 4.9 mm Hg. The vehicle for RSR13, namely saline, caused no change in medullary pO₂, which averaged 11.3 ± 4.0 mm Hg before saline and 11.7 ± 4.4 mm Hg after saline. Subsequent addition of mannitol did not alter medullary pO₂, which averaged 9.3 ± 4.2 mm Hg.
Fig. 4. 31P NMR spectroscopic measurements of inorganic phosphate (Pi, A) and adenosine 5' triphosphate (ATP; B) concentrations following administration of RSR13 after pretreatment with indomethacin alone (△, N = 8) or indomethacin + furosemide (●, N = 8). Data are expressed as fraction of baseline and shown as mean ± SEM. *P < 0.05 and **P < 0.01 vs. RSR13 following indomethacin alone.

**Renal 31P NMR studies**

In the rats given indomethacin followed by RSR13, the inorganic phosphate (Pi) concentration doubled. Furosemide prevented this change in Pi (Fig. 4A). Renal ATP content decreased by 40% following the infusion of RSR13 in indomethacin-pretreated rats (Fig. 4B). When rats were given indomethacin followed by furosemide and then RSR13, there was no fall in ATP content (Fig. 4B). A significant decrease of intracellular pH (pH_i) accompanied the decreases in ATP and increases in Pi in the RSR13 plus indomethacin-treated rats (6.71 ± 0.08 at 50 min vs. 7.26 ± 0.04 at baseline, P < 0.01), and furosemide treatment prevented this decrease.

**DISCUSSION**

This study employed a model of acute renal failure that is believed to mimic human acute renal failure and
has medullary hypoxia as its primary pathophysiological disturbance [6]. We originally hypothesized that, by “unloading” oxygen bound to hemoglobin, RSR13 would improve renal function in this model because tubular hypoxia is a hallmark of functional and morphologic injury. However, precisely the opposite effect was found: RSR13-treated rats developed a transient, short-lived, reversible renal dysfunction, whereas the control group given saline vehicle did not develop acute renal dysfunction. We hypothesize that the reason for the lack of acute renal failure in the saline vehicle-treated group was related to volume expansion. Heyman et al have shown that acute volume expansion prevents the development of ARF in an identical model [2]. The addition of approximately 2.5 mL of isotonic saline alone to the peritoneal cavity, which matched the volume of the RSR13 solution, was necessitated by the solubility characteristics of RSR13 [4]. Volume expansion or repletion is known to delay or attenuate the ischemic renal failure in both experimental animals and susceptible patients exposed to contrast agents [12].

Not only did creatinine clearance decrease in the RSR13-treated rats, but serum creatinine, BUN, and fractional excretion of sodium (FE\textsubscript{Na}) increased. These measurements are an indication of acute tubular dysfunction characteristic of reversible ischemic acute renal failure [13]. All renal parameters in the RSR13-treated rats returned to normal by the third day after radiocontrast exposure.

One explanation for this rather unexpected kidney response to RSR13, as compared with all other tissues, is that oxygen was released from hemoglobin in the most proximal portions of the outer medulla (the early portion of the descending vasa rectae), leading to a large decrease in pO\textsubscript{2} in the deeper nephron regions of the kidney. Such an effect would widen the difference between oxygen supply and demand (for transport-associated energy needs).

To confirm this hypothesis, direct measurements of oxygen tension were utilized at various sites within the kidney, as well as simulations that we had previously developed to predict gas tensions along the nephron.

Based on the simulations, RSR13 would be expected to increase, not decrease, local oxygen tensions if the vasa rectae blood flow remained high. However, if blood flow through the vasa rectae was substantially reduced (as might be expected after indomethacin administration and/or salt depletion), RSR13 was predicted to increase the amount of kidney tissue exposed to anoxia (Fig. 2). Our mechanistic studies found that the administration of indomethacin reduced the medullary pO\textsubscript{2} to essentially zero (Table 2) even without pre-existing sodium depletion and radiocontrast administration. We believe that this strongly supports the concept that the renal dysfunction in this model is due to RSR13-enhanced shunting of well-oxygenated blood from the descending to ascending vasa rectae in the setting of very low vasa rectae blood flow induced by indomethacin.

No attempt was made to measure oxygen tensions under these combined conditions because the spatial limitations of the oxygen electrodes would not allow us to confirm or refute this hypothesis. However, further support for this concept was provided with the \textsuperscript{31}P NMR studies that demonstrated very significant falls in ATP concentrations with reciprocal increases in inorganic phosphate concentrations (reflecting tissue hypoxia and an accompanying intracellular acidosis) following RSR13 administration after the administration of indomethacin.

It is important to note that when RSR13 was administered in the absence of indomethacin, there was essentially no deleterious effect on renal function noted (Fig. 1). Clearly, if there is not concomitant reduction of blood flow, as occurs with indomethacin, RSR13 would not be expected to be injurious to kidney function.

Through an appreciation of the unique vascular anatomy of the kidney and its role in the determination of gas tensions along the nephron, it appears that one could develop maneuvers by which the effects of RSR13 could be mitigated or minimized. The most attractive approach may reduce energy needs (and the imbalance between oxygen availability and energy consumption) by reducing “active” transport in the mTAL with a loop diuretic such as furosemide. A furosemide treatment-induced increase in medullary pO\textsubscript{2} has been convincingly demonstrated in rats as well as more recently in humans [2, 14–16]. The current study also demonstrated this effect of furosemide. Returning to the acute renal failure model, furosemide substantially attenuated the transient renal dysfunction produced by RSR13. Similarly, whereas RSR13 on the background of prostaglandin inhibition caused significant depletion of ATP, furosemide pretreatment completely prevented the ATP depletion.

Our findings are of interest for several reasons. On the theoretical side, the observations offer additional validation for the medullary hypoxia concept which was proposed by Epstein and colleagues in the mid-1980s [1], and they support the utility of using our simple model of gas exchange along the nephron to predict pathophysiological events. On a more practical note, the ultimate utility of RSR13 as a clinical agent to improve tissue oxygenation in other organs requires that effects on renal function be avoided. The clinical circumstances of hypoperfusion and shock where RSR13 could have great of utility in improving tissue oxygenation in the cardiac and cerebral vascular beds [4, 5], therefore, are circumstances in which renal dysfunction also might occur. It is interesting to note that transient renal dysfunction was recently reported in several patients undergoing general surgery who had received RSR13 [17]. We suggest that the surgery and/or attendant anesthesia might have decreased
renal blood flow, making these subjects more susceptible to RSR13-induced renal anoxia. Our data suggest that co-administration of RSR13 and furosemide (with close attention to preventing diuretic-induced volume depletion) in situations in which renal hypoperfusion is likely to be a comorbid event would prevent or substantially attenuate transient renal dysfunction.

In summary, RSR13 administered in the setting of volume depletion and prostaglandin inhibition caused transient renal dysfunction. This renal dysfunction did not complicate RSR13 administration in the absence of prostaglandin inhibition and was completely prevented by administration of a loop diuretic. The oxygen unloading effects of RSR13 may have deleterious effects on renal oxygenation and function under conditions where renal oxygenation is impaired.

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