Rapid Renal Clearance of Immunoreactive Canine Plasma Myoglobin

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SUMMARY Rates and mechanisms of myoglobin removal from plasma were examined in closed-chest dogs, using disappearance patterns of immunoreactivity and radioactivity after i.v. canine myoglobin radiolabeled with $^{125}$I. Arterial immunoreactive myoglobin concentration decreased monoexponentially over a 2-decade range, with rate constants of disappearance averaging $-0.080 \pm 0.014$ min$^{-1}$ (± SD) (corresponding to half-times of disappearance of 8.9 ± 1.5 min). Renal arteriovenous difference in immunoreactive myoglobin concentration documented extraction of the parent molecule, with extraction ratios averaging 0.36 ± 0.06. Renal venous specific activity increased a few minutes after myoglobin administration, consistent with discharge from the kidney of nonimmunoreactive radiolabeled peptides of the parent molecule. Arterial disappearance of $^{125}$I was subsequently delayed in relation to immunoreactive myoglobin. Urinary recoveries of immunoreactive parent molecule and radiolabeled constituents were limited, averaging 2.5 ± 1.1% and 12 ± 1.1% over a 6-hour period. Arterial rate constants of disappearance of immunoreactive myoglobin decreased markedly with decreases in renal perfusion produced by obstruction of renal arterial inflow. We conclude that myoglobin entering the vascular space is normally cleared rapidly by renal catabolism. Serum myoglobin concentration-time patterns during acute myocardial infarction directly reflect patterns of protein entry into the vascular space after release from injured tissue.

SERUM MYOGLOBIN concentration is elevated abnormally in the early phase of myocardial infarction in almost all patients seen within 12-14 hours of the onset of chest pain.$^{1-3}$ Elevations in myoglobin frequently precede elevations of creatine kinase (CK); Stone et al.$^4$ reported that 45% of infarct patients show increased myoglobin levels with normal CK levels at the time of admission. Elevated myoglobin levels peak earlier and return toward normal more rapidly than elevated CK levels. Myoglobin concentration-time curves sometimes show multiple peaks during the first several hours of apparently uncomplicated infarction.$^6$

Although the basis for these differing patterns of myoglobin and CK elevation has not been defined, a difference in a rate of removal from serum is an important possibility. CK disappearance from the intravascular space has been characterized.$^{10}$ Although similar information for myoglobin is not available, myoglobin disappearance rates may be faster than those for CK; the kidney may play an important role.$^{11-14}$ If it does, sequential measurements of serum myoglobin may be useful for defining patterns of protein entry into the intravascular space during ischemic syndromes. Patients in the early states of myocardial injury — who may be the ideal candidates for interventions intended to minimize myocardial injury — might be identified as those with a normal serum myoglobin level on admission or a rapidly rising serum myoglobin over an interval of a few hours.

The present study was designed to define rates and mechanisms of clearance from the circulation of a bolus of radiolabeled myoglobin administered intravenously. The studies capitalized on the sensitivity of recently developed radioimmunoassay techniques for quantifying plasma myoglobin concentration. The immunoassay also offered the opportunity to contrast changing patterns of plasma immunoreactivity and radioactivity, the former presumably reflecting disposition of the native molecule and the latter catabolic byproducts circulating within the vascular space as well as the native molecule.

Methods

Canine myoglobin for exogenous administration and preparation of anti-dog myoglobin antibody was purified in a fashion similar to that for human myoglobin.$^9$ Sequential steps included homogenization of canine myocardium in buffered saline, centrifugation and isolation of the supernatant, precipitation of protein with 50% ammonium sulfate, repeat centrifugation with dialysis of the supernatant against saturated ammonium sulfate, dissolution of the precipitate with subsequent dialysis against 0.02 M phosphate buffer (pH 6.0), passage through a column of carboxymethyl cellulose (to separate myoglobin-hemoglobin from other proteins), and gel filtration on sephadex G-100. The purity of the preparation was evaluated by disc electrophoresis on polyacrylamide gel.

Portions of the final product used for exogenous administration were radiolabeled with $^{125}$I using the chloramine T procedure. Other portions were used to raise anti-dog myoglobin antibody in guinea pigs and to generate typical standard curves in the eventual radioimmunoassay. Radiolabeled myoglobin for the immunoassay was also prepared with chloramine T, but using $^{131}$I as tracer. The assay procedure was similar to that reported for human myoglobin,$^8$ except that rabbit anti-guinea pig gamma globulin was used for separation of bound and free antigen. Standard curves and precision of replicate analyses were of the
same order of magnitude as reported for the human assay, with as little as 0.5 ng of canine myoglobin being detected in a 50-μl serum sample. Studies of the efficiency of large cyanogen bromide peptides in inhibiting the binding of labeled myoglobin to specific antibody indicated that 1000–10,000 moles of peptide were required to compete effectively with 1 mole of whole protein. Such data make it unlikely that peptides generated from the parent molecule in vivo could compete effectively for antibody binding sites in the immunossay.

Sixteen closed-chest mongrel dogs that weighed 15–38 kg were anesthetized with sodium pentobarbital and ventilated with a piston respirator; the room air inspirate was supplemented with oxygen to maintain arterial oxygen saturation > 92%. Supplemental anesthesia was administered as needed during the later stages of each study. Arterial catheters for blood sampling and pressure monitoring were inserted through the femoral or carotid arteries. An additional catheter for myoglobin administration was inserted into the inferior vena cava or right atrium from a femoral vein. Baseline arterial myoglobin levels were drawn at the completion of instrumentation, after systemic anticoagulation with heparin.

In the first 10 of the 16 dogs, about 7.5 mg of purified myoglobin radiolabeled with 125I was injected through the systemic venous catheter over 1-minute. One-milliliter arterial blood samples were collected immediately before myoglobin injection, at 2–10-minute intervals for the first 60 minutes after injection, and at 15–30-minute intervals for 5 hours thereafter. In six dogs, the bladder was drained continuously and blood was also sampled from a renal vein, using a catheter inserted from a femoral vein and positioned fluoroscopically. Plasma from all arterial and venous blood samples was analyzed for 125I radioactivity and immunoreactive myoglobin, as were aliquots of cumulative 20-minute urine samples.

In the remaining six dogs, arterial clearance of immunoreactive myoglobin was studied at three levels of renal perfusion. The dogs were prepared similarly, with addition of a left atrial catheter (inserted retrograde from a carotid artery) for injection of radiactive microspheres. Microsphere measurements of renal blood flow were performed using 15-μm spheres labeled with 111In, 85Sr and 89Y and arterial reference sample collection and observing precautions recently reviewed by Heymann et al. After an initial microsphere flow determination, 7.5 mg of purified myoglobin was injected into the vena cava and arterial blood was sampled over 60 minutes as described above. A catheter inserted through a femoral artery was then positioned fluoroscopically in one of the two renal arteries and renal inflow was obstructed by injection of about 3 ml of metallic mercury. A microsphere measurement of renal flow was repeated and an additional 7.5 mg of myoglobin was administered, with collection of arterial samples for the subsequent hour. The same procedure was repeated after a further reduction of renal perfusion by mercury embolization of the contralateral renal artery. At the completion of each set of studies, the dog was killed and both kidneys were removed, weighed and dissolved in hydrochloric acid over 24–48 hours. Aliquots of the resultant mixtures were analyzed for microsphere radioactivity; renal perfusion was calculated and expressed as ml/min/kg body weight.

In the analysis of data, baseline immunoreactive myoglobin concentrations before each myoglobin injection were routinely subtracted from values measured subsequently. Because baseline levels were ordinarily 1–2% of peak levels achieved after injection, data were analyzed over a 2-decade range. The appropriateness of mono- vs multieponential fits for immunoreactive myoglobin data was evaluated using the linearizing transformation, ln(y). A least-squares regression line (y = Ax + B) and second-degree polynomial (y = Ax^2 + Bx + C) were calculated for each set of transformed data; since the variance of untransformed values of y was constant, individual points were weighted by a factor of y^2. The reduction in sum of squares associated with the second-order fit was then tested against the mean-square remaining after second-order regression in an F test; a p value < 0.05 was the level at which a non-mono-exponential fit to the original data was justified.

Monoexponential rate constants of disappearance were taken as the slope of the linear regression of ln(y) on time. In dogs in which renal venous blood was sampled, arteriovenous myoglobin extraction was calculated as d(y)/(A-V)/d(t) (A), where A and V represent instantaneous arterial and venous myoglobin concentrations and t = 60 minutes.

Results

Baseline immunoreactive plasma arterial myoglobin concentrations after preliminary instrumentation were < 100 ng/ml in 13 of the 16 dogs (average 82 ± 73 ng/ml [± SD]). Peak levels obtained by extrapolation to time 0 of data collected during the first 10 minutes after initial myoglobin injections averaged 5100 ± 1600 ng/ml (range 1960–8000 ng/ml).

Rate constants of disappearance of immunoreactive myoglobin at normal levels of renal perfusion are listed in table 1. Since multieponential data fits could be justified in only three of 16 dogs, only monoexponential rate constants (k) are presented. These averaged −0.080 ± 0.014 min⁻¹, corresponding to half-times of disappearance of 8.9 ± 1.5 minutes. Less than 90 minutes was required for arterial immunoreactive myoglobin to fall below 1% of the concentration initially measured after myoglobin injection. In the six dogs in which renal flow was measured directly, the average value was 11 ± 3.4 ml/min/kg.

Representative arterial disappearance patterns for immunoreactive plasma myoglobin and 125I radio-

*Conclusions were identical when each original data set was fitted with one- and two-exponential models [y(t) = A exp(-kt) and y(t) = A exp(-kt) + B exp(-st)] using a Marquardt algorithm. The reduction in sum of squares associated with the two-exponential fit was tested similarly.
activity in two dogs are shown in figure 1. Immunoreactive myoglobin concentration decays in a monoexponential fashion. The $^{125}$I radioactivity deviates from a monoexponential pattern at about 15 minutes and decays appreciably more slowly than immunoreactivity thereafter. Findings were similar in all 10 dogs in which arterial immunoreactivity and $^{125}$I radioactivity were studied simultaneously.

Representative differences between arterial and renal venous immunoreactive myoglobin concentrations during the initial 60 minutes after myoglobin administration are shown in figure 2. Extraction ratios for the six dogs studied in this fashion ranged from 0.27–0.50 (average 0.36 ± 0.06). Figure 3 is a comparison of arterial and renal venous specific activities for myoglobin ($^{125}$I counts/min/ng immunoreactive myoglobin). Renal venous specific activity rises above arterial specific activity about 6 minutes after myoglobin injection and remains higher thereafter. Arterial specific activity rises perceptibly at about 15 minutes, corresponding to the deviation of the arterial $^{125}$I decay from a monoexponential. Similar patterns of specific activity were found in all six dogs.

Figure 4 illustrates cumulative urinary recovery of immunoreactive myoglobin and $^{125}$I. For the six dogs, the total amount of immunoreactive myoglobin recovered over 6 hours averaged 2.5 ± 1.1% of the original amount injected. Simultaneous recoveries of $^{125}$I averaged 12.0 ± 1.1%.

Arterial disappearance patterns of immunoreactive myoglobin at three levels of renal perfusion in one dog are illustrated in figure 5. Disappearance is prolonged noticeably as renal perfusion is reduced. Table 2 is a summary of values during reduced renal inflow. Monoexponential rate constants of disappearance averaged $-0.050 ± 0.022$ min$^{-1}$ after embolization of one renal artery (renal flow $= 5.0 ± 2.2$ ml/min/kg), and $-0.027 ± 0.015$ min$^{-1}$ after embolization of both renal arteries (renal flow $= 2.4 ± 1.9$ ml/min/kg). Relationships between monoexponential rate constants of disappearance and renal blood flow (RBF) at all levels of perfusion are shown in figure 6. The 18 data points have a correlation coefficient of 0.86 ($p < 0.01$) and a composite linear regression of $k = 0.0051 RBF + 0.014 (Y = 0.046, S_y \cdot x = 0.0571)$. Monoexponential data fits were statistically better than monoexponential fits in two dogs after embolization of one renal artery, and in all six dogs after bilateral embolization.

**Discussion**

The present findings document that myoglobin entering the vascular space is normally cleared rapidly, with a half-time of clearance for the parent $^{125}$I radioactivity in two dogs after i.v. injection of purified canine myoglobin radiolabeled with $^{125}$I. Individual values are expressed as percentages of the concentrations obtained by extrapolating data collected during the first 10 minutes after injection to time zero. Immunoreactive myoglobin decreases monoexponentially over a 2-decade range, with half-times of 9.8 and 8.5 minutes respectively. Corresponding rate constants of disappearance are $-0.071$ and $-0.082$ min$^{-1}$. Arterial radioactivity falls at a slower rate than immunoreactivity after the first 10–15 minutes, reflecting persistence in blood of nonimmunoreactive fragments of the parent myoglobin molecule.
munoreactive molecule of about 9 minutes. The consistent renal arteriovenous differences in immunoreactive myoglobin concentration indicate that the kidney is an important site of removal. Renal catabolism is confirmed by the increase in renal venous specific activity that occurs a few minutes after administration of radiolabeled myoglobin; the increase presumably represents discharge from the kidney of radiolabeled peptides of the parent molecule, which do not compete effectively for binding to antimyoglobin antibody in the immunoassay. The dichotomy in arterial disappearance patterns of $^{125}$I and immunoreactivity beginning about 15 minutes after myoglobin administration is consistent with accumulation in the intravascular space of radiolabeled fragments originating from the kidney. The limited urinary recoveries of parent myoglobin molecule and radiolabeled fragments suggest postglomerular reabsorption of both moieties, which, on the basis of the molecular weight of the parent molecule (18,000), are expected to be filtered into glomerular fluid to an important degree. The reductions in rate of immunoreactive myoglobin clearance associated with reductions in renal perfusion further support the primary role of the kidney in myoglobin clearance. Monoexponential disappearance rates and renal perfusion correlate reasonably well over a wide range of renal flows (fig. 6). The better fit of multieponential than monoexponential functions at lower flow rates presumably reflects an increasing role of nonrenal catabolic processes or extravascular exchange. When data after bilateral renal artery embolization were fitted with a two-exponential function, rate constants for the slow compartment averaged $-0.015 \pm 0.008 \text{ min}^{-1}$. These values are much less than the rate constants listed in table I and may indicate the order of magnitude of the speed of nonrenal clearance mechanisms for myoglobin.

In considering the role of the kidney in myoglobin clearance, the proportion of normal plasma disappearance attributed to renal clearance should be estimated. Taking blood volume as 92.5 ml/kg$^{80}$ and assuming a hematocrit of 40%, the average rate constant of disappearance ($-0.080 \text{ min}^{-1}$) indicates a total plasma clearance of 4.3 ml/min/kg. Although renal

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Arterial and renal venous immunoreactive myoglobin concentrations in a representative dog after administration of canine myoglobin. Renal extraction of myoglobin is apparent.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Specific activity of myoglobin (Mb) in arterial and renal venous blood after administration of canine myoglobin radiolabeled with $^{125}$I. The specific activity of renal venous blood begins to increase about 6 minutes after injection. Arterial specific activity increases later but remains lower than renal venous specific activity.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Cumulative urinary recoveries of immunoreactive myoglobin and $^{125}$I-radiolabel in a representative dog after administration of canine myoglobin radiolabeled with $^{125}$I.}
\end{figure}
extractions ratios and renal blood flow were not measured in the same dogs, the average values of these measurements (0.36 and 11.4 ml/min/kg) suggest that renal plasma clearance for a hematocrit of 40% is about 2.5 ml/min/kg, or about 60% of total plasma clearance. Although relatively crude, this estimate supports the concept that renal catabolism is, quantitatively, the major mechanism for disposal of circulating myoglobin. Serum levels at which normal renal clearance mechanisms become saturated remain to be determined.

These interpretations of our data are consistent with previous studies of clearance and catabolism of globin and with similar studies of previous studies of clearance and catabolism of myoglobin. Although relatively crude, this estimate supports the concept that renal catabolism is, quantitatively, the major mechanism for disposal of circulating myoglobin. Serum levels at which normal renal clearance mechanisms become saturated remain to be established.

**TABLE 2. Rate Constants of Disappearance of Immunoreactive Myoglobin in Arterial Blood During Reduced Renal Inflow**

<table>
<thead>
<tr>
<th>Dog</th>
<th>RBF (ml/min/kg)</th>
<th>k (min⁻¹)</th>
<th>RBF (ml/min/kg)</th>
<th>k (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>8.7</td>
<td>-0.093</td>
<td>5.2</td>
<td>-0.052</td>
</tr>
<tr>
<td>12</td>
<td>3.8</td>
<td>-0.051</td>
<td>4.2</td>
<td>-0.037</td>
</tr>
<tr>
<td>13</td>
<td>2.5</td>
<td>-0.042</td>
<td>0.8</td>
<td>-0.022</td>
</tr>
<tr>
<td>14</td>
<td>6.2</td>
<td>-0.043</td>
<td>2.1</td>
<td>-0.018</td>
</tr>
<tr>
<td>15</td>
<td>4.7</td>
<td>-0.031</td>
<td>1.4</td>
<td>-0.014</td>
</tr>
<tr>
<td>16</td>
<td>3.8</td>
<td>-0.042</td>
<td>0.7</td>
<td>-0.019</td>
</tr>
<tr>
<td>Mean</td>
<td>± 5.0</td>
<td>± 0.050</td>
<td>± 2.4</td>
<td>± 0.027</td>
</tr>
<tr>
<td>± SD</td>
<td>± 2.2</td>
<td>± 0.022</td>
<td>± 1.9</td>
<td>± 0.015</td>
</tr>
</tbody>
</table>

*Multiexponential fits were justified for dogs 13 and 16 after unilateral embolization and for all dogs after bilateral embolization.

Abbreviations: k = monoexponential rate constant of disappearance; RBF = renal blood flow.

**Figure 5.** Arterial disappearance of immunoreactive myoglobin after administration of canine myoglobin at three levels of renal blood flow.

**Figure 6.** Monoexponential rate constants of disappearance (k) of immunoreactive myoglobin as a function of renal blood flow.
tion of patterns of entry into the vascular space of protein released from injured myocardium (provided that noncardiac sources of myoglobin do not contribute importantly to the serum curves). Myoglobin's rapid clearance from serum also makes it an advantageous marker for detecting recurrent protein release during the course of infarction. Kagen and colleagues reported recurrent elevations of myoglobin concentration during apparently uncomplicated infarction, suggesting either episodic ischemic events, episodic release after a single initial injury (as with reperfusion), or a combination of both. Since myoglobin levels were measured by microcomplement fixation rather than radioimmunoassay, definition was less than optimal, particularly in the range of 25–200 ng/mL. Further studies with radioimmunoassay techniques are needed.

In any consideration of myoglobin levels, the immunologic identity of cardiac and skeletal muscle myoglobin must be considered. Because of this limitation in specificity, it has always seemed unlikely that serum myoglobin concentration-time curves would have practical value for "sizing" myocardial infarctions. At the same time, because of myoglobin's unusually rapid clearance from the vascular space, it is a useful marker for studying important related questions.

The limited urinary recovery rates of both immunoreactive myoglobin and 125I radiolabel in the present studies are consonant with experience in patients with acute infarction, but at variance with positive reports of myoglobinuria using precipitin and hemagglutination-inhibition techniques. These differences could be related to differences in specificity of the assay procedures.

The present findings during reduction of renal arterial inflow confirm that myoglobin clearance rates can be reduced substantially in the presence of renal dysfunction. Serum creatinine values of 7 mg% or greater have been associated with increased values of serum myoglobin. Studies of low-molecular-weight proteins, such as Bence-Jones proteins, indicate that these moieties are filtered to an important degree at the glomerulus, but then reabsorbed almost quantitatively and catabolized at the tubular level. Renal tubular dysfunction might be expected to be associated with increased urinary excretion of unaltered myoglobin, though not necessarily with a diminished rate of serum clearance. The latter would be affected adversely by situations associated with reductions in glomerular filtration or any nonglomerular uptake process also involved in myoglobin clearance. In normal man, assuming a serum myoglobin level of 30 ng/mL, a renal blood flow of 1500 ml/min and a renal extraction of 36%, one would estimate that the kidneys normally catabolize 23 mg/day of myoglobin. This value is substantially higher than the extrapolmonary turnover rate of 0.3 mg/day estimated by Hällgren et al. from serum concentration-time curves in acute infarction. In retrospect, the latter approach may have been confounded by continuing release of myoglobin during the period in which rate constants of disappearance were calculated.

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Circulation. 1982;65:1522-1528
doi: 10.1161/01.CIR.65.7.1522

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